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THE PRODUCTION OF INTERMEDIATE-WINGED APHIDS WITH SPECIAL REFERENCE TO THE PROBLEM OF EMBRYONIC DETERMINATION [1]

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Aphids which are intermediate in structure between the parthenogenetic and the gamic type have already been studied in relation to the order of embryonic segregation (Shull, 1930, 1931, 1933). More abundant and more easily produced at will are those intermediates which are structurally between the winged and wingless parthenogenetic types. They offer the same opportunity to examine certain features of the mechanism of development as do any other intermediates which can be artificially produced.

Partly to obtain large numbers of these winged-wingless intermediates for analysis, and partly in the hope that the method of obtaining them would throw some light on their nature, extensive experiments in which the environmental agents known or believed to modify the aphid cycle were combined in a variety of ways were carried out over a period of years. This paper describes the experiments, which involved a total of 530,133 aphids, of which 9,152 were intermediate-winged.

The Stocks of Aphids

Two independent strains of aphids of the species *Macrosiphum solanifolii* have been used. One was collected near Ann Arbor in the year 1923 and has been maintained parthenogenetically ever since. This strain was clone A of a former paper (Shull, 1932) until it experienced a "mutation" in the fall of 1929, in which its characteristics were greatly altered, becoming clone A′ of that paper. The other strain was collected in Ann Arbor in 1931; to date none of the results from this line has been published.

[1] Contribution from the Department of Zoölogy, University of Michigan.

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Since it has been shown that conditions affecting the aphids at least a generation in advance of the beginning of an experiment may modify the results of the experiment (Shull, 1935), four different stocks of each of the two strains were continuously reared. One was kept at a temperature of 24°, one at 14°, one at alternating temperatures (24° for 8 hours, 14° for 16 hours daily), and one at room temperature which fluctuated considerably. All were in continuous light which was not necessarily of uniform intensity.

The Method of the Experiments

In general a complete set of experiments would proceed as follows. From each of the eight stocks mentioned above a number of aphids, both winged and wingless, were drawn shortly before (or in a few cases just after) they became adult. Some of each type of female were placed at a temperature of 24°, others at 14°. In each of these temperatures some were kept in continuous light, others in intermittent light (8 hours light, 16 hours darkness). In addition, some aphids from each of the stocks except the one in room conditions were brought to room temperature for one generation prior to the beginning of the experiment; their offspring, having grown up at room temperature, were then reared in each of the various combinations of conditions just outlined.

It was never possible to do all of these things at once. However, all experiments involving one stock, and sometimes those from two of the stocks, could be done simultaneously. Since fairly consistent results were obtained from each treatment, it is unlikely that any serious error has been introduced by performing the various parts of an experiment at different times.

Effect of Continuous and Intermittent Light in the 1923 Strain

The results of these experiments can be presented only as total numbers of individuals of the several kinds produced under each combination of conditions. This is best done in tabular form. In branching tables, the contrasting conditions placed nearest the data are the ones whose effect is best shown, since pairs of adjoining numbers represent the contrast. Other contrasts are between numbers at some distance from one another, and are less readily seen. It will be necessary, therefore, to arrange the data in several ways to show the significant results.

In Table I the last column preceding the data (the fourth column) includes the two contrasted light conditions, namely, continuous and intermittent (8 hours of light, 16 hours of darkness). It is therefore the effect of light which is best shown by this table. To save space the

TABLE I

The percentages of winged and intermediate-winged offspring produced by both wingless and winged aphids of the 1923 strain in various combinations of light and temperature both during and before the experiments.

Stock parents came from	Temperature of parents preceding experiment	Temperature of parents during experiment	Light on parents during experiment	Offspring					
				From wingless parents			From winged parents		
				Total number of offspring	Percentage winged	Percentage intermediate-winged	Total number of offspring	Percentage winged	Percentage intermediate-winged
24°	24°	24°	Cont.	7,640	50.7	0.26	12,816	59.7	0.37
			8–16	5,832	32.5	3.23	6,777	37.0	4.01
		14°	Cont.	7,835	46.5	0.18	17,056	42.4	0.28
			8–16	5,592	16.6	5.17	17,335	19.2	6.35
	Room	24°	Cont.	2,522	47.4	0.12	2,261	40.0	0.04
			8–16	1,650	38.4	3.03	1,737	28.6	4.38
		14°	Cont.	2,387	62.7	0.08	2,301	32.2	0.22
			8–16	2,445	15.4	4.05	1,647	9.0	10.75
14°	14°	24°	Cont.	6,550	49.4	0.11	8,309	33.6	0.13
			8–16	5,240	47.4	5.42	7,085	20.5	2.87
		14°	Cont.	7,182	55.0	0.07	6,806	21.3	0.13
			8–16	5,307	20.4	8.95	4,084	11.5	11.75
	Room	24°	Cont.	880	51.1	0.11	3,329	42.4	0.24
			8–16	846	39.7	5.52	3,326	29.8	2.56
		14°	Cont.	1,238	50.8	0.00	3,630	29.6	0.08
			8–16	1,017	18.6	7.37	3,169	10.4	9.09
Alt.	Alt.	24°	Cont.	5,581	49.3	0.13	10,505	55.3	0.39
			8–16	5,547	39.5	2.94	9,205	43.7	4.20
		14°	Cont.	7,257	35.8	0.12	12,786	39.6	0.13
			8–16	5,702	13.0	7.87	8,975	12.9	10.92
	Room	24°	Cont.	2,902	44.9	0.21	4,094	35.8	0.07
			8–16	2,709	35.4	3.03	3,545	25.3	2.03
		14°	Cont.	3,225	44.6	0.09	4,650	34.1	0.04
			8–16	2,760	17.8	8.48	3,190	7.4	7.62
Room	Room	24°	Cont.	4,415	46.8	0.18	8,015	45.8	0.29
			8–16	3,912	41.1	2.94	6,821	30.3	4.31
		14°	Cont.	4,694	43.8	0.17	9,268	44.8	0.21
			8–16	3,049	13.9	5.77	7,263	10.8	9.54

actual numbers of individuals of the several kinds are not given; only their percentages of the total, which are essential to quick comparison, are indicated. Any one who wishes to know the numbers can readily calculate them from the total. Though this paper is concerned only with intermediate individuals, the percentages of normal winged aphids is also given for the sake of a comparison to be made on a later page.

From Table I it is clear that more intermediate-winged aphids of this strain were produced in intermittent light than in continuous. This is true for both wingless and winged parents, for every stock, and for all temperatures both before and during the experiments. That is, in every combination of other agents, intermittent light yielded more intermediates than did continuous light. Moreover, the differences are large ones; in no instance is the ratio less than 10:1, and in many sets of conditions the intermediates were 20 or even 50 times as numerous in intermittent as in continuous light. Evidently continuity or discontinuity of light is of major importance in the production of intermediates in the 1923 strain.

Effect of Current Temperature in the 1923 Strain

The results of the experiments with the 1923 strain are rearranged in Table II so as to put the temperatures used during the experiment (24° and 14°) in the fourth column, next to the data, thereby contrasting most sharply the effects of these current temperatures.

The last column of this table, relating to those reared in intermittent light, shows the most striking contrast. In every combination of other conditions, more intermediate-winged offspring were produced at 14° than at 24°. The differences are fairly large, the ratios ranging roughly from 1.3:1 up to nearly 4:1. These differences are obtained, however, only in intermittent light.

In continuous light (column 7), the differences are small and mostly in the opposite direction. That is, more intermediates are produced at 24° than at 14° (one exception). Though all the numbers are small, the fact that nearly all the differences are of the same sign can hardly mean other than that there is a significant reversal of the effect of temperature in continuous light as compared with the temperature effect in intermittent light.

Wingless and Winged Parents in the 1923 Strain

By rearranging the experiments with the two types of parents at the right of the branching table, as is done in Table III, whatever difference there is in the tendency of wingless and winged parents to produce intermediate-winged offspring is best shown.

TABLE II

The data of Table I rearranged to show the contrast between 24° and 14° temperature in the production of intermediate-winged aphids by the 1923 strain.

Nature of parents	Stock parents came from	Temperature of parents preceding experiment	Temperature of parents during experiment	Offspring: Produced in continuous light: Total number of offspring	Produced in continuous light: Percentage winged	Produced in continuous light: Percentage intermediate-winged	Produced in 8-hr. light, 16-hr. darkness: Total number of offspring	Produced in 8-hr. light, 16-hr. darkness: Percentage winged	Produced in 8-hr. light, 16-hr. darkness: Percentage intermediate-winged
Wingless	24°	24°	24°	7,640	50.7	0.26	5,832	32.5	3.23
			14°	7,835	46.5	0.18	5,592	16.6	5.17
		Room	24°	2,522	47.4	0.12	1,650	38.4	3.03
			14°	2,387	62.7	0.08	2,445	15.4	4.05
	14°	14°	24°	6,550	49.4	0.11	5,240	47.4	5.42
			14°	7,182	55.0	0.07	5,307	20.4	8.95
		Room	24°	880	51.1	0.11	846	39.7	5.52
			14°	1,238	50.8	0.00	1,017	18.6	7.37
	Alt.	Alt.	24°	5,581	49.3	0.13	5,547	39.5	2.94
			14°	7,257	35.8	0.12	5,702	13.0	7.87
		Room	24°	2,902	44.9	0.21	2,709	35.4	3.03
			14°	3,225	44.6	0.09	2,760	17.8	8.48
	Room	Room	24°	4,415	46.8	0.18	3,912	41.1	2.94
			14°	4,694	43.8	0.17	3,049	13.9	5.77
Winged	24°	24°	24°	12,816	59.7	0.37	6,777	37.0	4.01
			14°	17,056	42.4	0.28	17,335	19.2	6.35
		Room	24°	2,261	40.0	0.04	1,737	28.6	4.38
			14°	2,301	32.2	0.22	1,647	9.0	10.75
	14°	14°	24°	8,309	33.6	0.13	7,085	20.5	2.87
			14°	6,806	21.3	0.13	4,084	11.5	11.75
		Room	24°	3,329	42.4	0.24	3,326	29.8	2.56
			14°	3,630	29.6	0.08	3,169	10.4	9.09
	Alt.	Alt.	24°	10,505	55.3	0.39	9,205	43.7	4.20
			14°	12,786	39.6	0.13	8,975	12.9	10.92
		Room	24°	4,094	35.8	0.07	3,545	25.3	2.03
			14°	4,650	34.1	0.04	3,190	7.4	7.62
	Room	Room	24°	8,015	45.8	0.29	6,821	30.3	4.31
			14°	9,268	44.8	0.21	7,263	10.8	9.54

This difference, though sometimes striking, is not everywhere of the same sign. In 21 of the 28 sets of conditions the winged parents produced the more intermediate offspring, and most of the differences are of some size. Of the 7 exceptions to this rule, 6 are from parents which were reared at room temperature throughout their immature stages, suggesting that perhaps the peculiarly variable conditions thus introduced were favorable to intermediate-winged offspring. However, the seventh exception (which is the seventh pair in the left side of Table III) was exhibited by parents not reared at room temperature; and the difference shown here is one of the more striking ones in the table. It seems impossible to bring the wingless and winged aphids under any general rule concerning their tendency to produce intermediate-winged offspring.

Effect of Antecedent Temperatures in 1923 Strain

Two other rearrangements of the results of the experiments would be required to show most directly the effects of the temperatures applied to the aphids prior to the beginning of the experiments. One table would show any difference in the number of intermediate offspring produced by aphids taken from the several temperature stocks, that is, the stocks reared at 24°, 14°, alternating, and room temperatures respectively. The other rearrangement would show whether bringing the parents to room temperature through all their immature stages before using them in an experiment affected the number of their intermediate-winged offspring. The differences which these tables would show are not regular enough to warrant their presentation in that form. A statement of them, however, should be made.

Keeping the experimental parents at room temperature through their immature stages, when that represented a change from previous conditions (as it would for aphids taken from the 24°, 14°, and alternating-temperature stocks), caused them to produce fewer intermediate-winged offspring in 16 out of 24 combinations of other conditions. Of the 8 which showed the opposite effects, 6 were reared at 24° during the experiment, the other 2 were reared at 14°. Among these 8 likewise were aphids from each of the four temperature stocks. Again it is impossible to state a general rule describing the effect of a one-generation transfer to room temperature in advance of an experiment.

Still less regular is the difference between the various temperature stocks, in their tendency to produce intermediate-winged offspring. Aphids from each stock yielded more intermediates than did those from all the other stocks, in from one to three of the combinations of other conditions. This is true regardless of whether the aphids were brought

to room temperature for one generation before the experiment or not. Aphids changed from the alternating-temperature stock to room temperature yielded more than did those reared continuously at room tem-

TABLE III

Differences between wingless and winged parents of the 1923 strain in their production of intermediate-winged offspring under various conditions. (*ws* = wingless, *wd* = winged.)

Temperature of parents preceding experiment	Light on parents during experiments	Temperature of parents during experiments	Stock parents came from	Nature of parents	Total number of offspring	Percentage of offspring winged	Percentage of offspring intermediate-winged
24°	Cont.	24°	24°	*ws*	7,640	50.7	0.26
				wd	12,816	59.7	0.37
		14°	24°	*ws*	7,835	46.5	0.18
				wd	17,056	42.4	0.28
	8–16	24°	24°	*ws*	5,832	32.5	3.23
				wd	6,777	37.0	4.01
		14°	24°	*ws*	5,592	16.6	5.17
				wd	17,335	19.2	6.35
14°	Cont.	24°	14°	*ws*	6,550	49.4	0.11
				wd	8,309	33.6	0.13
		14°	14°	*ws*	7,182	55.0	0.07
				wd	6,806	21.3	0.13
	8–16	24°	14°	*ws*	5,240	47.4	5.42
				wd	7,085	20.5	2.87
		14°	14°	*ws*	5,307	20.4	8.95
				wd	4,084	11.5	11.75
Alt.	Cont.	24°	Alt.	*ws*	5,581	49.3	0.13
				wd	10,505	55.3	0.39
		14°	Alt.	*ws*	7,257	35.8	0.12
				wd	12,786	39.6	0.13
	8–16	24°	Alt.	*ws*	5,547	39.5	2.94
				wd	9,205	43.7	4.20
		14°	Alt.	*ws*	5,702	13.0	7.87
				wd	8,975	12.9	10.92

Temperature of parents preceding experiment	Light on parents during experiments	Temperature of parents during experiments	Stock parents came from	Nature of parents	Total number of offspring	Percentage of offspring winged	Percentage of offspring intermediate-winged
Room	Cont.	24°	24°	*ws*	2,522	47.4	0.12
				wd	2,261	40.0	0.04
			14°	*ws*	880	51.1	0.11
				wd	3,329	42.4	0.24
			Alt.	*ws*	2,902	44.9	0.21
				wd	4,094	35.8	0.07
			Room	*ws*	4,415	46.8	0.18
				wd	8,015	45.8	0.29
		14°	24°	*ws*	2,387	62.7	0.08
				wd	2,301	32.2	0.22
			14°	*ws*	1,238	50.8	0.00
				wd	3,630	29.6	0.08
			Alt.	*ws*	3,225	44.6	0.09
				wd	4,650	34.1	0.04
			Room	*ws*	4,694	43.8	0.17
				wd	9,268	44.8	0.21
	8–16	24°	24°	*ws*	1,650	38.4	3.03
				wd	1,737	28.6	4.38
			14°	*ws*	846	39.7	5.52
				wd	3,326	29.8	2.56
			Alt.	*ws*	2,709	35.4	3.03
				wd	3,545	25.3	2.03
			Room	*ws*	3,912	41.1	2.94
				wd	6,821	30.3	4.31
		14°	24°	*ws*	2,445	15.4	4.05
				wd	1,647	9.0	10.75
			14°	*ws*	1,017	18.6	7.37
				wd	3,169	10.4	9.09
			Alt.	*ws*	2,760	17.8	8.48
				wd	3,190	7.4	7.62
			Room	*ws*	3,049	13.9	5.77
				wd	7,263	10.8	9.54

perature in some combinations of conditions, but fewer in other combinations.

Antecedent temperatures on the whole thus give mixed results in the

production of intermediate-winged offspring. All these relations can be made out from the tables already given, though the numbers contrasted are not in adjoining lines. It seems not worth while to bring them thus together in view of the impossibility of generalizing from them.

Wing Production and Intermediate Wings in the 1923 Strain

It is worthy of note that in general the greatest production of intermediate wings does not accompany the greatest production of wings. Intermittent light, with every combination of the other factors, resulted in more intermediate-winged but fewer winged individuals, than did continuous light (Table I). Current temperature of 14°, with intermittent light, produced likewise more intermediate-winged but fewer winged offspring than did 24°, in every combination of other factors (Table II, right). However, current temperature of 14°, with continuous light, produced mostly fewer intermediate-winged and at the same time mostly fewer winged offspring than did 24° (one or two exceptions, Table II, left half). The differences here are mostly small, however, for both the winged and the intermediate-winged.

Within any one of these major groups, the relation between wing production and intermediate wings may also be determined from their correlation. This has been calculated for the two halves of Table II separately. For the right half of that table, where production of intermediate wings is everywhere high and that of normal wings moderate, the correlation between the two is found to be $r = -0.68 \pm 0.10$. This means that in general the fewer the wings the more numerous the intermediate wings. This negative relation is not due, however, to a mere conversion of wings into half-grown wings at low temperature. Even if all the intermediate-winged aphids were converted into winged ones, there would still be in most conditions a considerable deficit of winged individuals at the lower temperature.

For the left half of Table II the corresponding correlation between wings and intermediate wings is $r = 0.26 \pm 0.18$, which is positive but not of proven significance. There is no simple relation, therefore, between production of wings and production of intermediates; but on the whole these two things are oppositely affected by the same sets of conditions.

The bearing of these facts on the nature of intermediates is discussed in a later section.

The 1931 Strain of Aphids

The 1931 strain of aphids differs very considerably from the 1923 strain in the production of both winged and intermediate-winged individuals. The proportion of intermediate aphids in the 1931 strain is only about one-eleventh of that in the 1923 line. In wing production, the 1931 strain resembles the 1923 strain during the latter's first six years; that is, before its "mutation" of 1929. The 1931 line is thus more like clone A than like clone A′ of a former paper (Shull, 1932). Both clone A and the 1931 strain produced more winged individuals in intermittent than in continuous light; and when the relatively weak light used in the recent experiments is taken into account, the degree of their response to light is probably not very different.

Effect of Continuous and Intermittent Light on Intermediates in the 1931 Strain

The experiments with the 1931 strain are first presented with the contrast between continuous and intermittent light most plainly shown, as in Table IV. In 27 of the 28 pairs of percentages of intermediate-winged offspring the larger percentage is that obtained in intermittent light. The numbers are mostly small, but the fact that in all but one pair the differences are in the same direction indicates the significance of the result.

The only situation in which the difference is very marked is that involving a succession of 24°, room, and 14° temperatures; that is, in which aphids from the 24° stock were put at room temperature where their offspring grew to maturity, and these offspring became the parents in the experiment at 14°. Both wingless and winged parents responded in the same decisive way, hence the large number of intermediate offspring can hardly be accidental.

The one exception to the direction of difference arose from a situation like that above except that aphids from the 14° stock were used. The preceding temperature (room) and the current temperature (14°) were the same. The difference was small, and the reversal of its sign is found only among the offspring of winged parents; hence it is of questionable meaning.

Effect of Antecedent Temperatures on Intermediates in the 1931 Clone

By rearranging the results obtained with the 1931 strain as in Table V, the effect of temperatures applied to the ancestors of the ex-

TABLE IV

The percentage of winged and intermediate-winged offspring produced by both wingless and winged aphids of the 1931 strain in different combinations of light and temperature both during and before the experiments.

Temperature of parents during experiment	Stock parents came from	Temperature of parents preceding experiment	Light on parents during experiment	Offspring					
				From wingless parents			From winged parents		
				Total number of offspring	Percentage winged	Percentage intermediate-winged	Total number of offspring	Percentage winged	Percentage intermediate-winged
24°	24°	24°	Cont.	6238	11.5	0.06	6332	6.7	0.13
			8–16	5827	26.8	0.46	5055	18.8	0.69
		Room	Cont.	2094	32.8	0.00	1777	20.8	0.06
			8–16	2063	29.3	0.58	1659	25.8	0.60
	14°	14°	Cont.	6742	4.8	0.06	3411	1.0	0.09
			8–16	6169	33.9	0.24	3315	20.2	0.72
		Room	Cont.	2758	11.5	0.11	1824	4.6	0.00
			8–16	2913	32.4	0.27	1707	17.3	0.06
	Alt.	Alt.	Cont.	3720	7.2	0.05	5304	7.9	0.23
			8–16	3642	10.2	0.14	5144	22.5	0.45
		Room	Cont.	3945	12.4	0.03	1412	3.0	0.00
			8–16	2546	26.8	0.31	980	7.4	0.20
	Room	Room	Cont.	6709	21.0	0.18	6480	7.0	0.23
			8–16	5231	33.5	0.98	5214	18.4	0.98
14°	24°	24°	Cont.	6306	14.7	0.08	6587	10.7	0.08
			8–16	5187	75.7	0.42	5506	75.8	0.64
		Room	Cont.	2593	43.0	0.12	2086	39.8	0.10
			8–16	2196	53.6	2.46	1557	37.9	4.82
	14°	14°	Cont.	7356	9.4	0.05	3990	3.0	0.00
			8–16	6074	82.6	0.36	3381	67.7	0.62
		Room	Cont.	2874	12.6	0.14	1586	9.6	0.06
			8–16	2473	85.5	0.36	1712	84.0	0.00
	Alt.	Alt.	Cont.	4358	13.6	0.02	5735	3.1	0.04
			8–16	3774	89.1	0.19	5088	83.7	0.35
		Room	Cont.	4024	16.4	0.05	1441	3.3	0.00
			8–16	3134	86.8	0.10	1179	84.8	0.08
	Room	Room	Cont.	6463	14.6	0.11	6308	10.1	0.32
			8–16	6041	80.6	0.53	5012	75.1	1.06

TABLE V

The data of Table IV rearranged to show the effect of different antecedent temperatures applied to the parent aphids of the 1931 clone.

Light on parents during experiment	Temperature of parents during experiment	Stock parents came from	Temperature of parents preceding experiment	Offspring					
				From wingless parents			From winged parents		
				Total number of offspring	Percentage winged	Percentage intermediate-winged	Total number of offspring	Percentage winged	Percentage intermediate-winged
Cont.	24°	24°	24°	6238	11.5	0.06	6332	6.7	0.13
			Room	2094	32.8	0.00	1777	20.8	0.16
		14°	14°	6742	4.8	0.06	3411	1.0	0.09
			Room	2748	11.5	0.11	1824	4.6	0.00
		Alt.	Alt.	3720	7.2	0.05	5304	7.9	0.23
			Room	3945	12.4	0.03	1412	3.0	0.00
		Room	Room	6709	21.0	0.18	6480	7.0	0.23
	14°	24°	24°	6306	14.7	0.08	6587	10.7	0.08
			Room	2593	43.0	0.12	2086	39.8	0.10
		14°	14°	7356	9.4	0.05	3990	3.0	0.00
			Room	2874	12.6	0.14	1586	9.6	0.06
		Alt.	Alt.	4358	13.6	0.02	5735	3.1	0.04
			Room	4024	16.4	0.05	1441	3.3	0.00
		Room	Room	6463	14.6	0.11	6308	10.1	0.32
8–16	24°	24°	24°	5827	26.8	0.46	5055	18.8	0.69
			Room	2063	29.3	0.58	1659	25.8	0.60
		14°	14°	6169	33.9	0.24	3315	20.2	0.72
			Room	2913	32.4	0.27	1707	17.3	0.06
		Alt.	Alt.	3642	10.2	0.14	5144	22.5	0.45
			Room	2546	26.8	0.31	980	7.4	0.20
		Room	Room	5231	33.5	0.98	5214	18.4	0.98
	14°	24°	24°	5187	75.7	0.42	5506	75.8	0.64
			Room	2196	53.6	2.46	1557	37.9	4.82
		14°	14°	6074	82.6	0.36	3381	67.7	0.62
			Room	2473	85.5	0.36	1712	84.0	0.00
		Alt.	Alt.	3774	89.1	0.19	5088	83.7	0.35
			Room	3134	86.8	0.10	1179	84.8	0.08
		Room	Room	6041	80.6	0.53	5012	75.1	1.06

perimental aphids is shown. The last column preceding the actual data shows the temperature applied to just one generation prior to the beginning of the experiments. Since, however, there is some coupling of the temperature applied to that generation with the temperature of preceding generations (not all combinations having been tried), it is a combination of the last two (third and fourth) columns of the branching part of the table to which the data are most closely related.

This arrangement shows that aphids taken from the room temperature stock yielded more intermediate-winged offspring than did those of any of the stocks kept at other temperatures (24°, 14°, alternating), provided the stock conditions were maintained up to the very beginning of the experiment. This is true of both wingless and winged parents, and of every combination of other conditions, though one of the differences is so slight as not to appear as a difference in the table.

Changing the parents to room temperature from one of the other temperatures (24°, 14°, or alternating) yielded on the whole more intermediate-winged offspring than did continuing the stock temperature up to the beginning of the experiments, if wingless parents were used, but fewer intermediates if winged parents were used. There are, however, three exceptions out of twelve to each part of that statement. Moreover, one of the exceptions is one of the only two very striking differences to which attention was called in the preceding section. These exceptions, which do not themselves possess any one characteristic in common, weaken the generalization that seems otherwise warranted.

Effect of Temperature and of Nature of Parents on Production of Intermediates in 1931 Strain

The remaining factors produce too little or too variable an effect on intermediacy to warrant rearrangement of the data in tables to show those effects clearly. The following statements of these irregular effects can be verified from the tables already given, by comparing percentages not adjacent to one another.

The nature of the parents has no regular influence on the number of intermediates. Winged parents produced more intermediate offspring in 17 combinations of external conditions, but fewer in 11 combinations. Eight of the latter group of 11 came from 14° and alternating temperature stocks and in addition were at room temperature for one generation before the experiment started.

Current temperature had no consistent effect, since aphids at 14° produced more intermediates than did those at 24° in 13 combinations, but fewer in 14, and an equal number in 1. No single factor is common to all of the 13, nor to all of the 14.

Relation of Intermediacy to Wing Production in the 1931 Strain

As was stated earlier, the only situation in which the 1931 clone produced very many intermediate-winged individuals was intermittent light and a current temperature of 14°, applied to parents which came from the 24° stock but which were reared at room temperature throughout their own immature stages (eighteenth line, Table IV).

These conditions are not exceptionally conducive to production of normal-winged aphids. Winged individuals were, it is true, more abundant (47 per cent) under these conditions than in the experiments as a whole (34 per cent) in the 1931 strain. But other combinations of conditions resembling this one yielded higher wing production as often as lower. Thus, changing only the current light from intermittent to continuous served to reduce wing production 13 per cent; and changing only the current temperature from 14° to 24° reduced wing production 19 per cent. But omitting only the change to room temperature during the immature stages of the parents in the experiments increased wing production 29 per cent; and taking the parents from the 14° or the alternating temperature stock instead of the 24° stock increased wing production 38 per cent and 39 per cent respectively. None of these percentages appears directly in any of the tables, since they are based on the combined output of both wingless and winged parents.

It may also be pointed out that the set of conditions yielding the most intermediate individuals produced fewer winged ones than any other set of conditions that included a current temperature of 14°, but more than any set involving 24° temperature during the experiments.

On the whole, therefore, the greatest number of intermediate aphids would seem to be produced under circumstances which favored medium wing production in this strain.

Less connection between wings and intermediate wings is to be seen in a comparison of lines 17 and 18 in Table IV. The two very large percentages of intermediates are shown in line 18. The conditions resulting in the aphids of line 17 differ from those producing the aphids of line 18 only in that they included continuous light instead of intermittent light. This one change reduced the proportion of winged aphids only moderately, but reduced the intermediates very greatly. Continuous light reduces the number of winged aphids in some other clones (clone A, for example, Shull, 1932), and in general does so for the 1931 strain used in these experiments. That response is well shown in Table V, where the percentages of winged individuals are consistently lower in the upper half of the table than in the lower. But in that

upper half, the percentages in the ninth line stand out as far higher than any others. Continuous light had little of its usual effect on the number of winged offspring in this particular combination of other agents, but reduced the intermediate-winged individuals to as low a proportion as the average of all others in continuous light (upper half of Table V). It is possible, therefore, to change the production of intermediates without greatly changing the production of normal-winged aphids.

If the relation of intermediates to winged aphids is to be studied by the correlation method, the data of the 1931 strain must be broken up into three parts. Since intermittent light favors both wings and intermediate wings, strong correlation between the two would be shown by the data as a whole, but this would indicate nothing more than Table IV does. Current temperature also has a marked effect on wing production in intermittent light, though not in continuous light. Since calculation of correlation coefficients is justified only when the two variable qualities are distributed unimodally, and is most significant when the distribution is fairly symmetrical, separation of the data into three groups is necessary to avoid wide gaps and extreme skewness. The three groups are (1) those aphids reared in intermittent light and at 24°, (2) those reared in intermittent light at 14°, and (3) those reared in continuous light without distinction of temperature. From two of these have been omitted two percentages (winged aphids in one, intermediates in the other) because they were so high as to create the discontinuity which grouping the data was designed to prevent. These high percentages must have some special reasons with which correlation studies are not fitted to deal. The three coefficients of correlation between percentage of winged and percentage of intermediate-winged are, respectively, $.30 \pm .24$, $-.70 \pm .15$, and $.03 \pm .20$. Two are insignificant, the third strongly negative.

It seems clear, therefore, that whatever is inducing wing development in these more unified segments of the experiments is not particularly conducive to production of intermediates—is, indeed, somewhat opposed to it.

Conceivable Schemes of Stimulation

The general method of explaining intermediates proposed by Goldschmidt (1923 et al.) particularly for intersexes is perhaps applicable to aphids. It involves the assumption that something (a hormone) responsible for the development of structural characters is present in different concentrations at different times, usually in increasing concentrations with increasing age during the critical period of development.

Since the sexes start development under the influence of different groups of genes, and since they typically possess in the end quite different structures, it was natural to assume two hormones, one for each sex. Development of the individual began with one of these hormones in the ascendancy, but somewhere in the course of differentiation it was overtaken by the other hormone, the moment of passing being the "turning point." Structures determined before that point were like those of one sex, characters determined after that point were like those of the other sex.

It would be possible to make the same assumptions regarding intermediates between winged and wingless aphids. These aphids, however, are presumably genetically alike. Moreover, the difference between them lies almost entirely in the possession by the winged type of a group of characters which are wanting in the wingless. It is therefore simpler to represent the differentiating characters as dependent upon the concentration of a single substance in the critical period of development. No serious flaw would be created in the concept of the fundamental nature of the intermediates even if this representation should prove to be in error.

It is assumed for the purpose of this discussion that this one hormone varies in concentration during development in the general manner portrayed by Goldschmidt's curves, which were originally suggested, no doubt, by the course of other biological processes in developing organisms. In proper concentrations it will be regarded as a stimulus to the development of the differential characters of the winged type, though it could as well be considered an inhibitor. The unsegmented egg will be represented as possessing only a minimal quantity of this hormone, though it is conceivable that the egg might have a higher concentration than any later stage. Within these self-imposed limits we may imagine the influence which various situations would have on the frequency and nature of intermediates.

Differentiating Characters.—The winged female aphid differs from the wingless in having wings, three ocelli on the head, wing muscles in the thorax, and extra sensoria on the third segment of the antennæ (4–6 in the wingless, 15–18 in the winged). There are color differences, the third segment of the antennæ being blackish and the thorax brownish in the winged type, but these are subject to considerable variation and are not included in this discussion. It is the four structural characters named which are assumed to develop if a certain concentration of the stimulating hormone is attained early enough.

Sharply Defined Single Thresholds.—The line of demarcation between wingless and winged could be a sharply defined level of concentra-

tion of the hormone, such that any deficit, however slight, would leave the differentiating structures wholly absent, while any excess, however small, over this level would cause those structures to be fully developed. The simplest case would be that in which the same concentration of the hormone would stimulate the development of all four structures—wings, ocelli, wing muscles, and sensoria.

The time in development at which these structures have their fate (their presence or absence) decided could likewise be a sharply defined moment, such that if the stimulus were applied in advance of that time the structures would develop, while if the stimulating level of the hormone were attained even only slightly after that time the organs would be entirely lacking in the adult. The simplest case is again that in which the time of determination is the same moment for all four of the structures.

This situation is represented in Fig. 1, *A*, in which time during development is expressed by horizontal distances and concentration of the hormone by vertical distances. The curves indicate the rate of accumulation of the hormone during development. Any curve passing to the left of and above the dot in the middle of the chart would be that of a winged adult; any curve below and to the right, that of a wingless adult.

Intermediate individuals would be practically excluded by the relations here described, since very seldom would a curve of concentration pass exactly through the intersection of stimulation level and time of determination.

Different Times of Determination.—The preceding situation could be modified by having different times of embryonic determination for the four structures, but the same concentration of the hormone as a stimulus for all. This condition is represented in Fig. 1, *B*. The order of determination of the four differentiating features is arbitrarily assumed purely for purposes of illustration. All curves of hormone concentration rising rapidly enough to pass the stimulating level in advance (to the left) of all these times of determination would be those of winged aphids; while all curves reaching that level to the right of (after) all the times of determination would be those of wingless individuals. Curves between these limits would lead to mosaics. The second curve in *B* would be that of an aphid having ocelli, wing muscles and extra antennal sensoria fully developed, but lacking wings entirely. The third curve would pertain to one lacking wings and ocelli altogether, but having wing muscles and extra sensoria fully present. The fourth curve would belong to an aphid having extra antennal sensoria to the full number, but lacking all the other marks of a winged individual.

It is proposed in this paper to use the term mosaic for a patchwork of typical parts, the word intermediate for an individual in which one

or more structures are partially developed. "Typical parts" means, in wingless aphids, the absence of any degree of the marks of a winged aphid. A mosaic thus would have one or more of the features of the winged aphid completely developed, but lack the others entirely. One individual could be both mosaic and intermediate, by having certain structures either fully developed or wanting, other structures partially developed.

The conditions described in *B* would lead to a certain number of mosaics, but rarely to intermediates. How many mosaics there were would depend partly on the lengths of the intervals between the times of determination.

Different Levels of Stimulation.—The several features of a winged aphid could be stimulated to develop by different concentrations of the hormone. If there were a single time of determination for all four of these features, the general situation would be as shown in Fig. 1, *C*. Without further explanation it should be clear that the second and third curves are those of mosaics, the second possessing ocelli, wing muscles and extra sensoria, the third having only wing muscles and sensoria. Intermediates would be rare, and the number of mosaics would depend on the extent of the differences among the levels of stimulation.

Range of Stimulation.—It seems likely that a certain concentration of the hormone could not act suddenly to determine a complete structure. More probably a given concentration, if applied early enough, serves to stimulate a slight development of the structure, while a greater concentration would cause a greater development of it. There would thus be a level (concentration) of beginning differentiation, and a level of complete differentiation. If this range of stimulation were identical for all four of the features of the winged aphid, and if there were a sharp time of determination likewise common to all four of them, the conditions would be represented by Fig. 1, *D*. Under these conditions there could be some intermediates, but no mosaics. Any individual whose hormone concentration rose above the level of beginning differentiation before the time of determination, but did not rise as high as the level of complete differentiation until after that time, would be an intermediate. All four of the distinguishing structures would be only partially developed. They might not be developed to equal degrees, since they might respond at different rates to increasing quantities of the hormone. The second curve in *D* would belong to an aphid having wings, ocelli, wing muscles, and antennal sensoria highly, but not completely, developed, the third curve to one in which these structures were only slightly differentiated.

How many intermediates were produced would depend in part on

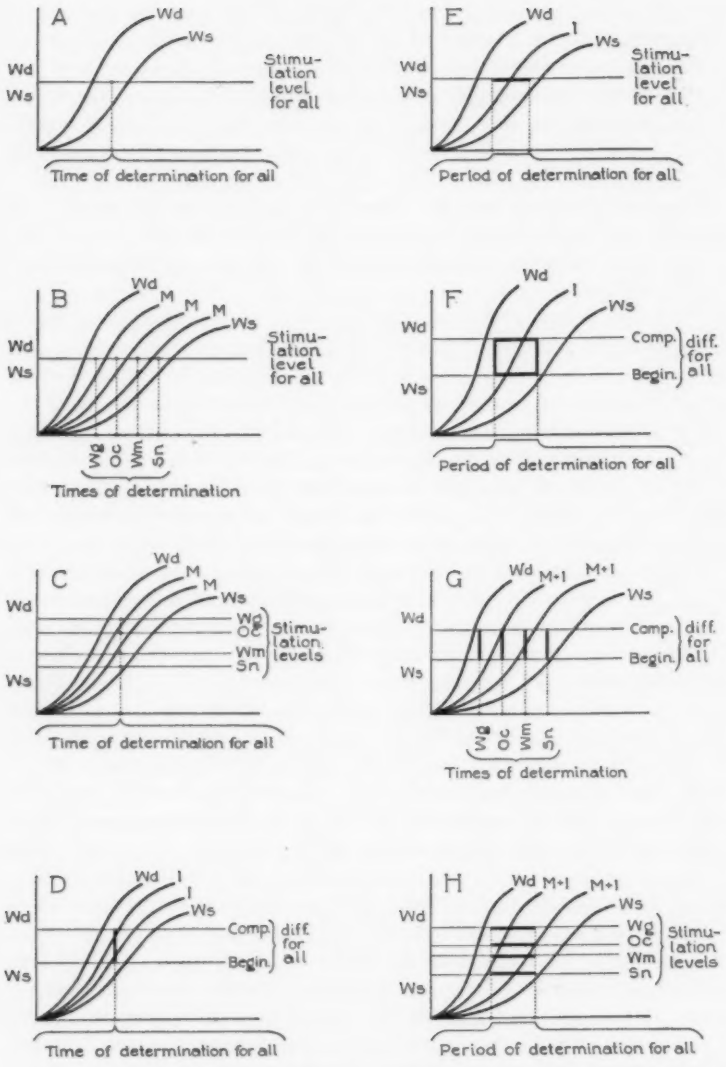

FIG. 1. Curves representing hormone concentration in relation to production of intermediate-winged individuals in aphids, under various assumptions concerning times or periods of determination and levels or ranges of stimulation during embryonic development. *I*, intermediate; *M*, mosaic; *Oc*, ocelli; *Sn*, sensoria; *Wd*, winged; *Wg*, wings; *Wm*, wing muscles; *Ws*, wingless.

the difference between the concentration which would begin and that which would complete the differentiation.

Period of Determination.—It is not necessary that a structure have its fate irrevocably fixed at one moment in development. That determination may be spread over a period of time. If the hormone rises to a stimulating concentration just before that period ends, the structure may be supposed to be slightly developed. If the hormone reaches a stimulating level soon after the beginning of the period of determination, the structure would be highly but not completely developed. In Fig. 1, *E*, it is assumed that there is one period of determination for all structures and one sharp level of stimulation for all of them.

The above situation would lead to the production of some intermediates, but no mosaics. The middle curve of *E* would be that of an aphid having perhaps half-developed wings, ocelli, wing muscles, and extra sensoria. How many intermediates there were would depend on the length of the period of determination.

Range of Stimulation and Period of Determination.—There may conceivably be a range of stimulation levels (as in *D*) and also a period of determination (as in *E*). The simplest such situation is one in which all four differentiating structures would be stimulated by the same range of concentrations of the hormone and be determined during the same period of time. It is represented in Fig. 1, *F*. Any curve of hormone concentration which passed through the rectangle formed by the intersection of the range of stimulation and the period of determination would be that of an intermediate. All the differentiating structures of such an individual would be partially developed, though not necessarily to an equal extent. There would be no mosaics. How many intermediates were produced would depend partly on the size of the rectangle —the extent of the range of stimulation and the length of the period of determination.

Range of Stimulation with Different Times of Determination.—If there be one range of stimulating concentrations of the hormone common to all four of the structures, and sharply defined times of determination which are different for all four, the conditions are those portrayed in Fig. 1, *G*. Any curve cutting one of the heavy vertical lines would belong to an individual intermediate for the structure whose time of determination and range of stimulation that heavy line represents. The same individual would be mosaic for any structures whose heavy line its curve did not cut. Thus, the second curve in *G* would be that of an aphid lacking wings, having almost complete ocelli, and having fully developed wing muscles and sensoria. The third curve would belong to an individual lacking wings and ocelli, but having partially developed wing muscles and fully developed sensoria.

Individuals not typically winged or wingless, produced under the above conditions, might well be both mosaic and intermediate. Some of them would perhaps be mosaic alone, especially if the range of stimulation were narrow and the times of determination were widely spaced. Most of them would be intermediate only, if the range of stimulation were broad and the intervals between times of determination were very short.

Common Period of Determination with Different Levels of Stimulation.—Another possibility is that all structures have the same period of determination but different sharply defined levels of stimulation, as in Fig. 1, *H*. An individual would be intermediate for a given character if its hormone curve cuts the heavy line representing its period of determination and level of stimulation. It would be mosaic for (either possess or lack) any structure whose corresponding heavy line it did not cut.

Intermediates would prevail if the period of determination were long and the stimulation levels close together. Mosaics would be more common if the period of determination were short and the levels of stimulation far apart. Many atypical individuals would doubtless be both mosaic and intermediate.

One Level of Stimulation with Distinct Periods of Determination.—A single sharp level of stimulation for all distinguishing structures may be combined with periods of determination which are different for the different characters. If these periods do not overlap, the situation is as pictured in Fig. 2, *I*. The second curve in *I* is that of an individual with partially developed wings and the other three features of a winged aphid fully formed. The third curve is that of a mosaic having wing muscles and sensoria fully developed but wholly lacking wings and ocelli. Intermediates would prevail among atypical individuals if periods of determination were long and close together; mosaics would lead in numbers if periods of determination were short and separated by wide intervals.

Single Time of Determination with Separate Ranges of Stimulation.—If all the differentiating structures were determined at the same moment, but each had its own range of stimulation which did not overlap any of the others, the situation would be that represented by Fig. 2, *J*. The second curve of this chart, cutting one of the heavy lines representing time of determination and range of stimulation but missing the others, represents a mosaic and intermediate individual. The third curve, missing all the heavy lines, is that of a mosaic only. Intermediates would be favored, among atypical aphids, by wide ranges of stimulation close together; mosaics would be favored by narrow ranges widely spaced.

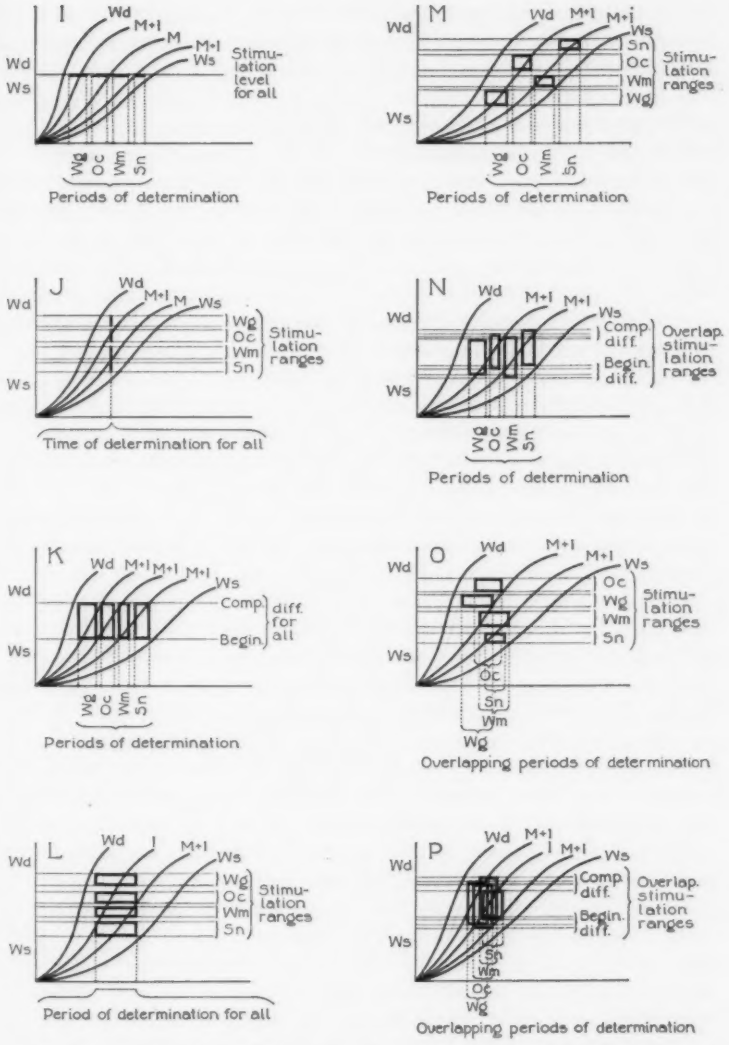

FIG. 2. Continuation of Fig. 1, representing increasing substitution of ranges for levels of stimulation, and of periods for times of determination, and increasing overlapping in place of distinct ranges and periods.

One Range of Stimulation with Distinct Periods of Determination.—If stimulation occupied a range common to all structures, and each organ had a different period of determination without overlapping, Fig. 2, *K*, would represent the conditions. The three curves in the middle are those of both mosaics and intermediates, since each curve cuts one or more of the heavy rectangles and misses one or more others. If the rectangles were narrow and high and close together laterally (that is, if periods of determination were short and not far apart while the threshold of complete differentiation were far above the threshold of beginning differentiation), most atypical individuals would be intermediate only. If periods of determination were short and widely separated, and the range of stimulation were narrow, mosaics would predominate.

Common Period of Determination with Distinct Ranges of Stimulation.—The situation indicated by this title is portrayed in Fig. 2, *L*. Individuals would be intermediate for those characters whose rectangles their hormone curves traverse, mosaic for those whose rectangles are missed altogether. If the common period of determination were long, and the ranges of stimulation were narrow and close together, most atypical aphids might well be intermediate only. In most other situations of this general kind, combined mosaics and intermediates would be more likely.

Distinct Periods of Determination and Separate Ranges of Stimulation.—In Fig. 2, *M*, is shown the combination of different periods of determination and different ranges of stimulation, without any overlapping of either periods or ranges. Many possibilities for either few or many intermediates, few or many mosaics, are presented by this situation. If the structures having early determination have low stimulation ranges, and those determined late have high stimulation ranges, so that the rectangles are arranged along a line rising obliquely to the right (as is more or less true in *M*), and if the curve of concentration of the hormone follows approximately this same course, the curves would cut most of the determination-stimulation rectangles if they cut any of them, and mostly intermediates would result. An atypical individual might easily be intermediate for all the distinguishing characters. If, however, structures determined early had high ranges of stimulation, and those determined late had low ranges of stimulation, so that in the figure the rectangles of determination and stimulation were arranged along a line sloping downward to the right, cutting the curve of hormone concentration nearly at a right angle, mosaics would prevail if the rectangles were small, and mosaic-intermediates would be most common if the rectangles were large. Many other relations between the period of determination and range of stimu-

lation could exist, such that the rectangles would be distributed irregularly in relation to the hormone curve. Each of these sets of relations would result in a particular frequency of mosaics or intermediates or both.

Distinct Periods of Determination with Overlapping Ranges of Stimulation.—The opportunity for pure mosaics would be reduced by having the ranges of stimulation overlap. This condition along with distinct periods of determination, is shown in Fig. 2, *N*. Unless the overlapping of ranges were very slight, it would be difficult for the hormone curve to pass between rectangles. While curves near the winged or the wingless extreme might miss some of the rectangles, they could scarcely miss all of them and still belong to atypical individuals.

Overlapping Periods of Determination with Distinct Ranges of Stimulation.—Equally difficult, with the preceding situation, for the production of pure mosaics would be the condition in which the periods of determination overlap while the ranges of stimulation are separate. In Fig. 2, *O*, where this situation is represented, there might appear to be a greater chance for one of the atypical curves to miss all the rectangles than in *N*, but that is only because the periods of determination have been assigned a smaller spread than was given to the ranges of stimulation in *N*. Most atypical aphids produced under these conditions would have to be intermediate with respect to some characters, and could easily be intermediate for all their distinguishing marks.

Overlapping of Both Periods of Determination and Ranges of Stimulation.—Still more favorable to intermediates, as compared to mosaics, than any of the preceding arrangements is that in which both the periods of determination and the ranges of stimulation overlap, as in Fig. 2, *P*. If the limits of the several periods and the several ranges did not differ very greatly, there would be few atypical curves that did not traverse several of the determination-stimulation rectangles, and many would traverse all of the rectangles. Intermediates would thus predominate over mosaics, though individuals both intermediate and mosaic could be fairly common.

The above are not all of the conceivable combinations of stimulation and determination. They will suffice, however, to illustrate the possibilities farther than there is any present possibility of forming judgments from them.

Nature of the Production of Atypical Forms

Since the factors which govern morphogenesis are as yet only very imperfectly understood, the possibilities outlined in the preceding sec-

tion may all be taken into account. What information, if any, concerning them is furnished by the methods of producing the intermediates?

Analysis of the characters of the atypical aphids has only begun, and it is too early to attempt to draw from them the conclusions to which complete study may lead. Already, however, it is clear that most if not all of them are intermediate in one or more respects. To what extent they may also be mosaic is not at present known, but very few of them are pure mosaics.

This fact should mean that the first few situations represented in the curves of Fig. 1 are less likely to exist than are the later ones or those of Fig. 2. Since sharply defined times of determination and levels of hormone stimulation would lead to mosaics, the facts so far ascertained favor the existence of a spread in one or both of these elements. Either there must be a difference between the concentration of the stimulating hormone which will lead to slight development of a structure and the concentration which will complete it, or the amount of development must depend in part on how long the effective stimulation is applied, or both of these statements must be true. Only a more complete knowledge of individual intermediates will indicate how much spread is required.

Until some physiological basis of embryonic determination [2] in the aphids is discovered, either a range of stimulating concentrations or a gradual process of determination may be postulated to explain intermediate individuals. Which is the more likely, or are both probable?

One fact reported in this paper which appears to bear on this question is the large difference in the capacity of the two strains used to produce intermediates. The 1931 line produced less than one-tenth of the number of intermediates produced by the 1923 strain. Also, this capacity to produce intermediates must be subject to large modifications, since the high capacity of the 1923 line (clone A′, Shull, 1932) arose suddenly by a "mutation" from a stock (clone A) which was producing apparently no more atypical individuals than the 1931 strain now produces. Which of the possible bases of intermediacy is more likely to exhibit large differences in different strains, or more likely to be subject to considerable change?

[2] The word "determination" is used in this paper despite its obvious defects. Since different events occurring at different times may be necessary antecedents of a morphogenetic change, a structure could not be said to be actually determined until the last such event had taken place. A morphological feature may be determined, as far as one agent is concerned, later than it is determined in relation to another agent. Were it possible, in the aphid experiments, to designate a single agent in relation to which determination was being considered, a better term that would not be too cumbersome could be devised. But the tables presented in this paper indicate that a complex set of circumstances is involved. Any substitute for the word determination that would be an improvement upon it would be unwieldy. Biologists are not likely to be misled, in this connection, by the briefer term.

Intermediacy could be made more frequent by increasing the length of the period or periods of determination, or by increasing the range of stimulation, so that more curves of concentration of the hormone would pass through them. It could also be made more abundant by bunching the curves of concentration; that is, by creating a set of conditions which would cause larger numbers of individuals to develop their stimulating hormone at approximately the same rate, which would have to be a rate that would make the curves of concentration traverse the lines or rectangles representing periods of determination or ranges of stimulation or both. This concentration of the hormone curves in a narrow band would have to occur in one strain of aphids, but not in another subjected to the same combination of light and temperature, or any other combination yet tested.

The second of these postulates, the clustering of the rates of hormone development, involves an increase in the measure of control exerted by the animals over the process of development. A strain of aphids in which more individuals developed in the same way would be exercising more regulation than would a line in which hormone increase was more nearly random. Now, the 1923 strain, which is the one in which such clustering of rates of hormone development would have to occur, does not regulate wing production nearly as precisely as does the 1931 line. Wing development responds to light conditions much more definitely in the latter than in the former, as a glance at the respective tables shows. The fact that the effect of intermittent light is reversed in the two strains is not at issue; the significant point is that the effect is much more precise in the 1931 strain than in that of 1923. It seems scarcely likely, therefore, that the control over rate of hormone concentration would be greater in the older line than in the newer one.

If this conclusion is justified, there is left the extent of the period of determination or of the range of stimulating concentrations of the hormone, or both, to account for intermediates. Nothing in the experiments here reported appears to provide a choice as between these two possibilities. However, embryonic development is known to consist in some animals, at least in part, of a chain of events in which some or all of the events provide the stimuli leading to later events. To whatever extent this is true, it would be difficult to imagine any great extension of the periods of determination of structures. Were many of these to be extended, and were each to wait upon the culmination of some preceding event, embryonic development would be correspondingly prolonged. Nothing is known that would render it probable that the embryos which become intermediate or mosaic adults take longer to develop, though the numerous ovarioles would make it possible for that to happen and for

the young aphids to be borne out of their regular turns based on the time of starting cleavage. On the whole, it would seem unlikely that embryonic development is retarded greatly by the factors leading to intermediacy.

The concentration of the hormone, on the contrary, is subject to no such known limitation. So far as anyone knows, the level of stimulation of completed differentiation could be farther above the level of beginning differentiation in one line of aphids than in another. So far as known there is no chain of events involved in the concentration of the hormone, such that one structure cannot begin to be stimulated to develop before the stimulation of another is completed.

It is suggested as more probable, therefore, that intermediacy in these aphids depends upon a gap between the thresholds of stimulation of beginning and of complete differentiation, rather than upon periods of determination or clustering of the rates of hormone development.

How this conclusion is related to the differences in the number of intermediate individuals induced by environmental conditions is difficult to ascertain. If the general concept that hormone concentration and range of stimulation levels combine to determine the winged type is correct, higher proportions of intermediates could be produced at the expense of winged aphids by lowering the hormone curves (reducing the rate of accumulation of the hormone) or at the expense of wingless aphids by elevating the hormone curves—all on the assumption, made to simplify the discussion, that the hormone is a stimulant rather than an inhibitor of the development of the characters of the winged females. The more striking changes in the number of intermediates produced by light and temperature in the 1923 strain were in general of a sign opposite to that of the change in the number of typical winged females produced by the same conditions. The suggestion contained in this comparison is that intermediates were being produced at the expense of winged adults. The reduction of the winged ones was in general, however, several times as great as the increase of the intermediates. In other words, many winged aphids were being converted into wingless ones as well as some into intermediates. This fact is not opposed to the supposition that hormone accumulation was being retarded; but since there were many wingless in most environmental conditions, a mere lowering of hormone curves would not necessarily leave any more of them in the intermediate zone. It would seem necessary to suppose that, in the lowering of the hormone concentrations, there was either an accumulation of the curves in the intermediate zone, or a widening of the ranges of stimulation so that more curves would intersect them.

One reason for not assuming an accumulation of hormone curves in the intermediate regions has already been pointed out.

It is worthy of note that in the 1931 strain, the only set of conditions which induced many intermediates served to increase, rather than diminish, the number of winged individuals (from wingless parents) or had practically no effect on the number of winged ones (from winged parents). If the explanations discussed above were to have general validity, it would be necessary to suppose that the intermediates from wingless parents were being produced at the expense of wingless ones, which might be due in part to a general lifting of the hormone curves. This contrast in the behavior of the two strains recalls their different responses to intermittent light in the production of wings; intermittent light produces fewer winged aphids in the 1923 strain, but more winged ones in the 1931 strain. There may be some common basis for these two differences. What reversal would be required in the hormone scheme to account for these contrasts it is impossible to say with any high degree of probability.

Whether intermediates are in any way dependent on the *direction* of some physiological change (that is, winged to wingless, or wingless to winged) can hardly be judged from the experimental methods of producing them. Analysis of the characters of the intermediates will be needed for an answer to this question.

Summary

Two strains of aphids were subjected to various combinations of temperature and light. In one strain (collected 1923) distinctly more intermediate-winged females were produced in intermittent than in continuous light, in every combination of other conditions. Among those reared in intermittent light, more intermediates were produced at a temperature of 14° than at 24°, in every combination of other conditions. Those reared in continuous light, however, produced usually fewer intermediates at 14° than at 24°, and the differences were small. In general, winged parents produced more intermediate offspring than did wingless parents, but there was much irregularity of this relation. Greatest production of intermediates and greatest production of typical winged females did not occur at the same time; there was, indeed, a tendency for the two frequencies to be opposed to one another. Other relations in this strain were rather indefinite. In the second strain (collected 1931) more intermediates were usually produced in intermittent than in continuous light, but the differences were small. More intermediate offspring were produced by parents drawn from a stock kept at

room temperature than by parents taken from stocks kept continuously at 14° or 24°, or from a stock regularly alternated daily between 14° and 24°, though again most of the differences were small. The greatest (and the only large) number of intermediates in the 1931 strain were produced by taking aphids from a stock kept at 24°, changing them for one generation to room temperature, then putting this latter generation, when adult, at 14°. In general, in this strain the greatest frequency of intermediates occurred when the frequency of typical winged aphids was medium. Other relations in the 1931 strain were not striking.

If it be assumed that production of the characteristics of winged aphids be dependent on suitable concentrations of a hormone which increases in amount during development (other assumptions could be made), intermediacy or mosaicism might result from various combinations of times or periods of determination, or of levels or ranges of concentration of the stimulating hormone, or both. Ranges of stimulating concentrations are less subject to known limits than are periods of determination, and are held to be the more likely, inasmuch as strains of aphids differed greatly in their tendency to produce intermediates. Intermediacy, as contrasted with mosaicism, is favored by extended periods of determination and ranges of stimulation, especially if there is overlapping of these periods and ranges for the several structures.

In part, the intermediates may replace winged aphids in the 1923 strain, and wingless ones in the 1931 strains.

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THE LOPING OF LAND-SNAILS

G. H. PARKER

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A little more than three decades ago Carlson (1905) published a very circumstantial account of the locomotion of a Californian land-snail, *Helix dupetithouarsi,* which may be described as a lope. The animal by appropriate muscular contractions lifts its head well above the substrate, projects it forward much in advance of its former position, and brings it down again on the surface over which it is progressing. Thus the anterior third of the snail forms an arch of which the head is one base and the mid-region of the foot the other, the part between being lifted well off the ground and representing the region of principal muscular action. This arch makes up one step in the snail's forward locomotion and passes over its foot and body in an antero-posterior direction to disappear at the tail. According to Carlson as many as three such arches may be seen progressing as waves over the snail at once. This lope results in unusually rapid progression and is employed by the animal in place of its ordinary slow creeping as a means, for instance, of escaping from an enemy.

In 1911 I called attention to this remarkable form of progression as described by Carlson and expressed the opinion that in the lope the large waves were probably combined with the waves for slow locomotion, a point not touched on by Carlson. I also pointed out that the loping waves took what has been designated as a *retrograde* course, that is, they ran from anterior to posterior, whereas in helices generally the ordinary locomotor waves are *direct,* from posterior to anterior. To clear up the question of the possibility in snails of a double set of waves, one opposite in direction to the other, and to elucidate further matters connected with so exceptional a scheme of progression, called for an examination of living specimens of *Helix dupetithouarsi* which at that time was impossible for me.

During a period of work at the Kerckhoff Laboratories of the California Institute of Technology in the spring of 1936, I noticed in the early morning on the brick walks of the campus interrupted snail trails of the kind figured by Carlson (1905) for this loping species. I immediately made a search for the snails and soon found living specimens in the act of making broken trails. The loping waves on these snails

ran from anterior to posterior as described by Carlson. The maximum number of them seen on the foot at once was three, the foot itself being about 4.5 cm. long. When the snail was in full action the arches of the body were so high that light from the early morning sun could be seen shining under them. These arches at times tended to become obliterated toward the hind end of the foot and in some instances they ceased after having passed over only the anterior half of the pedal surface, but as a rule they continued with full vigor all the way to the hind end. As a result of these differences the trails made by the snail varied from a succession of isolated spots through a series of connected bead-like spots to a continuous trail of uniform width. Such variations were to be noted in different parts of the single trail of a given individual, showing that from time to time the snail changed its type of locomotion.

A number of these snails were brought into the laboratory for further and closer study. Here they were made to creep upon large plates of glass where they could be induced to exhibit the same loping locomotion that they had shown on the damp brick paths. A single loping wave was found to pass over the foot from anterior to posterior, a distance of about 4.5 cm., in from eight to ten seconds. The snail while loping covered about ten centimeters in four minutes.

With the snail creeping on a glass plate it was possible to observe the foot from below and to record its action. In ordinary creeping the foot was covered by a succession of small transverse waves, about six in all, which passed from posterior to anterior and coursed over the length of the foot in from five to six seconds each. These both in direction and in general character agreed with the type of locomotor wave long since described for the majority of helices. When the snail began to lope, the large waves were easily followed from below the glass plate. Though the direction which they took over the foot was the opposite of that taken by the smaller waves, the two systems ran simultaneously and without interference. Thus the underside of the foot showed at once a system of small pedal waves running from behind forward, direct waves, and another of large loping waves running from anterior to posterior, retrograde waves. As a result of this double system the animal moved forward rapidly. It is hardly necessary to remark that the direction in which any such set of waves runs is independent of the direction in which the part of the foot that is concerned with the locomotor step moves. In both sets of waves the portion of the foot that is helpful in locomotion moves anteriorly; in the loping waves this locomotor action begins at the anterior end of the snail's body and foot and proceeds posteriorly and in the ordinary creeping waves it begins in the posterior part of the foot and proceeds anteriorly. The musculature

involved in the two systems of locomotion must, of course, be distinct; that for loping must include the body musculature directly dorsal and lateral to the foot and that for ordinary creeping the musculature immediately in the foot itself and next its creeping sole. Of the scores of instances of loping that I have observed from the underside of the glass plate, I have never seen the two systems of waves in any other relation than that just described. Further, I have never seen a snail in motion that has not exhibited the system of small waves. These are an invariable accompaniment of locomotion. When loping is undertaken, it is always superimposed on the smaller wave-system though without disturbance. Loping never occurs except after the snail has begun ordinary creeping. Thus the question left open by Carlson as to the relation of these two types of locomotion receives its answer.

I preserved the shells of the snails with which I worked in Pasadena and on my return to Cambridge, Massachusetts, I referred them to Mr. W. J. Clench of the Museum of Comparative Zoology for identification. Much to my surprise he informed me that they were the shells of *Helix aspersa,* a common European snail which was known to have been introduced into California some forty years ago and specifically into the region about Los Angeles. On looking up its habits I found in Taylor's monograph of British land and freshwater mollusks (1914) a brief statement with figures attributed to L. E. Adams showing that the English representatives of this species were known to lope.

This discovery led me to suspect that possibly Carlson in his original investigation had really worked on *Helix aspersa* and not on *Helix dupetithouarsi.* However, through the kindness of Mr. Clench I received a number of living *Helix* (now *Epiphragmorpha*) *dupetithouarsi* from Cypress Point, Monterey County, California, and I had the opportunity of testing these snails as I had done with *Helix aspersa.* They loped as *H. aspersa* did and I could confirm all the statement made by Carlson for *H. dupetithouarsi.* Moreover, this species, like *H. aspersa,* always showed the ordinary locomotor waves when it crept and never loped except when these waves were present. In both species, then, loping appears to be a form of locomotion literally superimposed on ordinary creeping and a means of accelerating progression beyond that attainable by ordinary methods.

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THE PHYSIOLOGY OF DIGESTION IN PLANKTON CRUSTACEA

II. Further Studies on the Digestive Enzymes of (A) Daphnia and Polyphemus; (B) Diaptomus and Calanus

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(From the Limnological and Physiological Chemistry Laboratories of the University of Wisconsin)

Introduction

It has been concluded by numerous workers previously quoted by the author (1935), in addition to Gellis and Clarke (1935) and Dehn (1930), that plankton Crustacea feed on particulate food and derive no nourishment from dissolved organic matter, or at least absorb so little of it that dissolved food stuffs account for only a small part of their metabolic needs. This circumstance makes it necessary that these organisms possess an enzyme system adequate for complete digestion of particulate food. Our first studies established the presence of a proteinase, amylase and lipase in *Daphnia*. This paper extends these studies to the peptidases of *Daphnia* and to the proteinases, amylases and lipases of *Polyphemus,* a gymnomerous cladoceran, and to the copepods *Diaptomus* and *Calanus*. Certain experiments to study the behavior of *Daphnia* proteinase at low temperature are also included.

Preparation of Extracts

Daphnia pulex were netted from Lake Monona, Wisconsin, in May 1935 when a pure culture was obtainable in large quantities. A pure culture of *Polyphemus* sp. was obtained from Crystal Lake, Wisconsin, in August 1934. *Diaptomus* sp. was found fairly abundant in a bog lake near Trout Lake, Wisconsin, in July, 1935. *Calanus finmarchicus* was collected from Buzzards Bay, Massachusetts, through the facilities of the Woods Hole Oceanographic Institution.

The glycerol extracts were made from the acetone-dried and petroleum ether-extracted whole organisms after grinding. The water extracts of *Daphnia* were prepared from the fresh, ground organisms and after filtration preserved with toluol.

Peptidases of Daphnia

Our criterion of the presence of dipeptidase was the splitting of leucylglycine, as pointed out by Waldschmidt-Leitz (1931). Some contention exists as to what was called aminopolypeptidase (Johnson et al, 1936), but for convenience this enzyme was considered present when the substrate leucyldiglycine was split, and carboxypolypeptidase was considered present when the substrate chloracetyltyrosine was attacked.

Titrations were made by the Linderstrøm-Lang (1927) method in 90 per cent acetone, using N/10 alcoholic HCl and naphthyl red as indicator.

No attempt was made to purify or separate the peptidases. The crude extract was used in all cases.

Table I

Hydrolysis of Peptides by Crude Extract

Enzyme	Substrate	pH	Hydrolysis* of one linkage in cc. of HCl		
			3 hrs.	12 hrs.	19 hrs.
Dipeptidase	*dl*-leucylglycine	7.2	0.08	0.28	0.54
Aminopolypeptidase	*dl*-leucyldiglycine	7.2	0.15	0.42	0.55
Carboxypolypeptidase	chloracetyl-l-tyrosine	7.2	0.03	0.11	——

* Hydrolysis of one linkage of one component is indicated in the two *dl* mixtures.

The enzyme-substrate mixture for dipeptidase determination consisted of 5 cc. of N/10 leucylglycine in phosphate buffer pH 7.2, and 2 cc. of 1 per cent *Daphnia* water extract. This was incubated at 34° C. and at intervals 2 cc. of the digest was removed and titrated. An HCl titration figure of 0.71 cc. was equivalent to 100 per cent hydrolysis of one linkage of one component. Table I shows the progress of hydrolysis in cc. of HCl.

For aminopolypeptidase 4 cc. of N/10 leucyldiglycine, and 2 cc. of 1 per cent water extract and 1 cc. of water were incubated at 34° C. Two cc. aliquots were withdrawn at intervals and titrated. An HCl titration figure of 0.57 cc. was equivalent to 100 per cent hydrolysis of one linkage of one component. Hydrolysis is indicated in Table I.

A limited supply of substrate for determination of carboxypolypeptidase prevented more than one analysis. Four cc. of M/30 chloracetyltyrosine, 2 cc. of water extract and 1 cc. of water were incubated at 34° C. Two-cc. samples were titrated at intervals.

It is perhaps true that the activity of these water extracts of *Daphnia* was considerably less than the activity of the enzymes *in vivo*. Several experiments on tissues and secretions of the hog and dog indicated that the activity of the enzyme preparation was in the order: (1) secretion, (2) extract of fresh tissue, (3) extract of dry tissue.

Effect of Temperature on Daphnia *Proteinase*

Daphnia is active in lake waters of low temperature. In fact they are known to spend the major part of the day in the hypolimnion of stratified lakes, where the temperature is as low as 4° to 8° C. The question arises as to whether their enzymes are particularly adapted to activity at low temperatures. In order to test this, extracts of a warm-blooded animal (hog) were tested at 20° and at 8° C. and the depression of activity compared with a similar depression of activity of *Daphnia* proteinase.

TABLE II

Activity of Daphnia and hog proteinase at 20° and 8° C. The results are expressed in milligrams of trichloracetic acid soluble N in 20 cc. of filtrate.

Digestion time	Temperature			
	20° C.		8° C.	
	Daphnia	Hog	Daphnia	Hog
hours				
1	0.28	6.41	0.00	3.23
2	0.81	10.78	0.29	5.28
5	2.56	16.78	0.93	8.90
13	4.18	——	1.78	——
24	6.01	——	2.22	——

One gram of dried, defatted hog pancreas was mixed with 100 cc. of 50 per cent glycerol. After standing at room temperature for 24 hours the mixture was filtered through silk bolting cloth. Ten cc. of this filtrate were added to 125 cc. suspension of casein in water (10 gm./100 cc. H_2O) and kept at 20° C. Twenty-five-cc. samples were removed at intervals and precipitated with 35 cc. of 10 per cent trichloracetic acid. The suspended protein was filtered off and the total nitrogen (Kjeldahl) was determined in duplicate on 20 cc. of the filtrate.

A similar digest was set up at 8° C. and the same procedure carried out. The bath consisted of running Lake Mendota water whose temperature was 8° C. at the time of the experiment.

Glycerol extracts of dried, defatted, ground *Daphnia* were prepared by extracting 1.0 gm. of *Daphnia* with 100 cc. of 50 per cent glycerol. This extract was treated in the same manner as described above for hog pancreas.

After 2 hours digestion (Table II), the hog pancreas produced 48 per cent more nitrogen at 20° than it did at 8° C. *Daphnia* proteinase produced 36 per cent more nitrogen at 20° than at 8° C. In 5 hours the respective percentages were 53 and 36. From these data it appears that *Daphnia* proteinase behaves, in general, like that of the hog and follows the van't Hoff law, so that it possesses no super-activity at a low temperature.

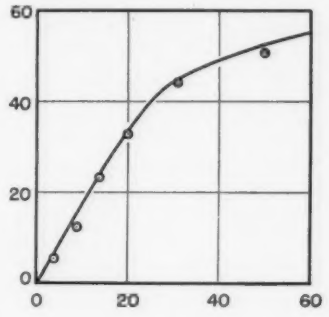

FIG. 1

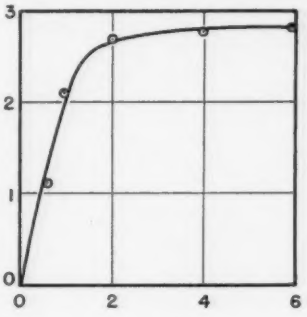

FIG. 2

FIG. 1. Hydrolysis of gelatin at pH 7.2 by a proteinase of *Polyphemus*. The ordinate represents decreasing viscosity (ΔV); the abscissa is the time in minutes.

FIG. 2. Hydrolysis of starch by an amylase of *Polyphemus*. The ordinate is the number of cc. of N/40 $Na_2S_2O_3$, or the amount of N/40 reducing groups; the abscissa is the time in hours of incubation at 37° C.

SOME ENZYMES OF POLYPHEMUS

Proteinase.—A glycerol extract was made from comminuted, dried and defatted *Polyphemus*. One part was diluted to 0.025 per cent for use in proteinase determination and another to 1 per cent for amylase and lipase determination. To 0.5 cc. of 0.025 per cent glycerol extract was added 5 cc. of 1.5 per cent Sargent's gelatin; the mixture was incubated at 34° C. and the viscosity periodically measured in an Ostwald viscosimeter by the method of Northrop (1922). The proteinase was designated as a tryptic enzyme, for it was highly active at pH 7.2, almost inactive at pH 4.35 and completely inactive at pH 3.0. The curve in Fig. 1 indicates the nature of the enzyme activity on gelatin at pH 7.2.

Amylase.—Polyphemus extract (1 per cent) was also capable of hydrolyzing starch. Twenty-five cc. of 3 per cent starch at pH 7.2, 25 cc. of water and 5 cc. of 1 per cent glycerol extract were incubated at 37° C. At intervals 10-cc. samples were withdrawn and the reducing groups determined iodometrically according to Baker and Hulton (1920). The results are shown in Fig. 2.

Lipase.—Four per cent tributyrin was attacked by an esterase of the extract. Fifty cc. of 4 per cent tributyrin emulsified with sodium glycocholate and 5 cc. of 1 per cent glycerol extract were placed at 37° C. Ten-cc. aliquots were withdrawn and titrated with N/20 NaOH. The titration figures can be read from Fig. 3.

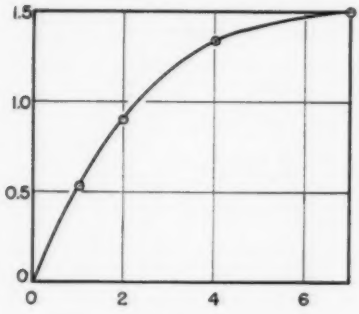

FIG. 3

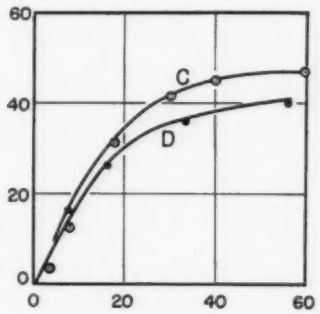

FIG. 4

FIG. 3. Hydrolysis of tributyrin by lipase of *Polyphemus*. The ordinate represents the number of cc. of N/20 NaOH; the abscissa is the time in hours.

FIG. 4. Hydrolysis of gelatin at pH 7.48 by proteinases of *Diaptomus* (*D*) and *Calanus finmarchicus* (*C*). The ordinate represents the decreasing viscosity of the gelatin (ΔV); the abscissa is the time in minutes.

SOME DIGESTIVE ENZYMES OF DIAPTOMUS AND CALANUS

The purpose of this study was to analyse extracts of a marine and a fresh water copepod for enzymes which attack proteins, carbohydrates and fats. An attempt was made with *Calanus* to corroborate the results of Bond (1934) by the use of different extraction and assay methods.

Clarke (1934) has adequately discussed the problem of marine copepod nutrition and has (1935) presented good evidence that their food, as in *Daphnia*, is of a particulate nature. The work of Bond represents a study of some of the enzymes of the marine copepod *Calanus finmarchicus*. Alcoholic extracts that he made were able to hydrolyze gelatin, starch and ethylbutyrate.

Materials

The marine copepod *Calanus finmarchicus* and the fresh water copepod *Diaptomus* sp. were collected at the places mentioned in an earlier division of this paper.

Methods of extraction were identical with those described above for hog and *Daphnia*.

Examination of the Extract

Proteinase.—A proteinase that hydrolyzed gelatin was demonstrated in the extract of *Calanus* and *Diaptomus* by the viscosity method. On the basis of the *Daphnia* proteinase unit (1 unit: $20\Delta V/20$, Hasler,

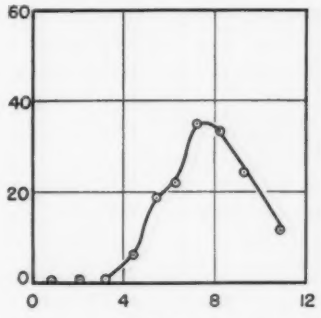

FIG. 5

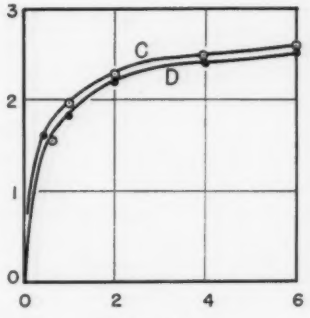

FIG. 6

FIG. 5. The pH activity curve of *Calanus* proteinase on gelatin. The ordinate represents decreasing viscosity of the gelatin (ΔV); the abscissa represents pH.

FIG. 6. Hydrolysis of starch by amylases of *Diaptomus* (*D*) and *Calanus* (*C*). The ordinate represents the number of cc. of N/40 $Na_2S_2O_3$, or the amount of the N/40 reducing groups; the abscissa is the time in hours of incubation at 37° C.

1935), 0.5 cc. of 1 per cent glycerol extract of dried, defatted *Diaptomus* contained 1.5 units. A similar amount of *Calanus* extract had a content of 1.7 units. One per cent extracts of *Daphnia* had 1.5 units. The amounts of enzyme in these extracts appear to be of the same magnitude. The curves in Fig. 4 show that digestion took place within the first few minutes of enzyme activity.

Sufficient extract was available from *Calanus* to construct a pH activity curve. Figure 5 shows the enzyme to be of definite tryptic type with maximum activity between pH 7–8. Inactivity resulted when the substrate pH was reduced to pH 3.5. Medium activity was evident

between pH 4–6 which indicated the presence of a katheptic or autolytic enzyme of the tissues. The alcoholic extracts of *Calanus* made by Bond (1934) were most active at pH 8.0–8.49. The secondary optimum of his extracts at pH 3.6–4.0 were unconfirmed in this work. The secondary optimum resulting from the autolytic enzymes was pH 5.6. The discrepancy of pH optima may be due to variations in extracting technique, for Mansour-Bek (1932) found that crude proteinase extract of *Maja squinado* had a pH optimum on gelatin of 6.0; the optimum for purified enzyme was pH 8.1. The pH of the buffered substrates used in this experiment was determined with the aid of the glass electrode.

Amylase.—Both *Diaptomus* and *Calanus* extracts contained active amylase which hydrolyzed starch. The iodometric titration previously

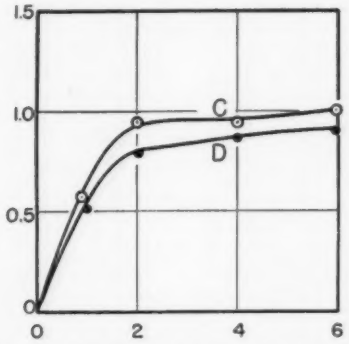

FIG. 7. Hydrolysis of tributyrin by lipases of *Diaptomus* (*D*) and *Calanus* (*C*). The ordinate indicates the number of cc. of N/20 NaOH; the abscissa is the time in hours.

described was used as a criterion of starch hydrolysis. The amounts of N/40 $Na_2S_2O_3$ present in 10 cc. of digest can be read from Fig. 6. These figures are equivalent to the amount of N/40 reducing sugars liberated by hydrolysis. In both instances 5 cc. of 1 per cent glycerol extract were added to 25 cc. of 3 per cent starch at pH 7.2 and 25 cc. of water and incubated at 37° C. At intervals 10-cc. samples were withdrawn and the reducing groups titrated.

Lipase.—Tributyrin was attacked by an esterase present in both *Diaptomus* and *Calanus* extracts. Fifty cc. of 4 per cent tributyrin were emulsified with sodium glycocholate to which were added 5 cc. of 1 per cent extract of *Diaptomus;* extracts of *Calanus* were examined by the same procedure. At intervals 10-cc. aliquots were withdrawn and titrated with N/20 NaOH. The results are shown in Fig. 7.

Experiments in all instances were duplicated and simultaneously run with adequate controls.

Discussion

Biochemical analyses presented in Table I, and by the author in a previous paper (1935), demonstrate that *Daphnia* possesses a system of proteolytic enzymes which can attack a protein (in this case gelatin and casein) and cleave it to amino acids. This bears out the contention that plankton crustaceans can utilize particulate organic matter.

The experiments on temperature effect on enzyme activity show that the *Daphnia* and hog enzymes follow, in general, the van't Hoff rule that a reaction approximately doubles for every 10° C. increase in temperature. It suffices to conclude from these data, that a low temperature does reduce the entire metabolism of the organism. More data should be collected on this subject, for Wiersma (1928) held that *Astacus* amylase was strongly active at 0° C., while Yonge (1926) found that enzymes of *Ostrea* were inactive at this temperature.

Another cladoceran (*Polyphemus*) and a copepod (*Diaptomus*) have been added to the list of plankton Crustacea which can be definitely said to possess functional digestive enzymes capable of attacking the three important food stuffs. It is quite clear from the review of Krüger (1933) and Yonge (1931) that these entomostracans together with *Daphnia* do not differ from the enzyme types of the larger Crustacea such as *Astacus* and *Maja*.

Although this work concludes the long series of experiments designed to solve the problem of nutrition in plankton Crustacea as challenged by Pütter, there remains an untouched field in enzyme chemistry of Crustacea, namely the kinetics and purification of these enzymes. A study of the kinetics of purified enzymes has been made by Mansour-Bek (1932) on the decapod, *Maja squinado*. She brought to light some interesting similarities between vertebrate and invertebrate enzymes, e.g. purified proteinase was activated by mammalian enterokinase. A continuation of her stimulating work would be an excellent contribution. Difficulties may be expected, however, in working with entomostracans, for the present purification methods require large amounts of extract to carry out proper elution.

Appreciation is expressed to Professor H. C. Bradley for furnishing laboratory facilities and for gladly proffering biochemical advice; also to Professor C. Juday for biological suggestions and personal interest in the problem.

SUMMARY

1. Three peptidases (dipeptidase, aminopolypeptidase and carboxypolypeptidase) were found in water extracts of *Daphnia*. The presence of these and a proteinase (author, 1935) demonstrate that *Daphnia* can completely utilize a protein.

2. Proteinases of *Daphnia* were no more active than those of the hog pancreas at a temperature of 8° C. Both enzyme systems follow, in general, the van't Hoff rule.

3. Proteinases, amylases and lipases were found in glycerol extracts of *Polyphemus* and *Diaptomus*. All proteinases were of the tryptic type with an optimum activity at pH 7–8. They simulate *Daphnia* enzymes and show that plankton Crustacea have complete enzyme mechanisms for digestion of particulate food.

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SYMMETRY AND REGULATION IN MNEMIOPSIS LEIDYI, AGASSIZ[1]

B. R. COONFIELD

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The ctenophores, a group in which *Mnemiopsis* is included, are usually referred to as biradially symmetrical animals. By some they are believed to have primarily a radial symmetry. There is of course the idea of bilateral symmetry expressed in the term biradial. As one sees this animal swimming freely in water it does seem to possess a radial symmetry. This impression is received because its organs are distributed about a central, longitudinal axis. But a close examination of this animal reveals that most of its organs are paired and are located on two opposite sides of the central, single organ, the stomodeum (Figs. 1 and 11). A method other than and including the study of the anatomy of this animal has been used by me in getting information about its symmetry. This information was obtained during a series of experiments which had been planned to show other characters of this animal. The one feature which has been demonstrated repeatedly during experimentation on *Mnemiopsis* is the power to regulate itself. This power to regulate its body following injury either in nature or caused by experiments seems to have some bearing on symmetry. I propose, therefore, to show in this report that *Mnemiopsis* possesses a particular type of symmetry, bilateral symmetry without dorso-ventrality, as well as the power to regulate itself, and that in this animal symmetry and regulation are associated with each other.

Experiments

The operations were performed on the specimens and each was kept in a separate finger bowl which was partly immersed in running water. Observations, drawings, and photographs were made at frequent intervals. Only a very few of the experimental animals failed to survive. The changes observed during the reorganization following the operations were recorded in detail. In a great many instances these changes were observed quite easily with only the aid of glasses of low magnification. The drawings were made with the aid of a camera lucida while the photographs were taken with a Leica camera with an extension tube outfit.

[1] Contribution No. 19 from the Department of Biology, Brooklyn College.

Although the experiments listed and described in this report seem to be quite varied they can be grouped under two headings; experimental cuttings, and experimental graftings.

Experimental Cuttings

The animals were cut across at four levels of the body and only the oral pieces were retained for observation. By this cutting at different levels some of the oral pieces lost only the very apical tip of the animal

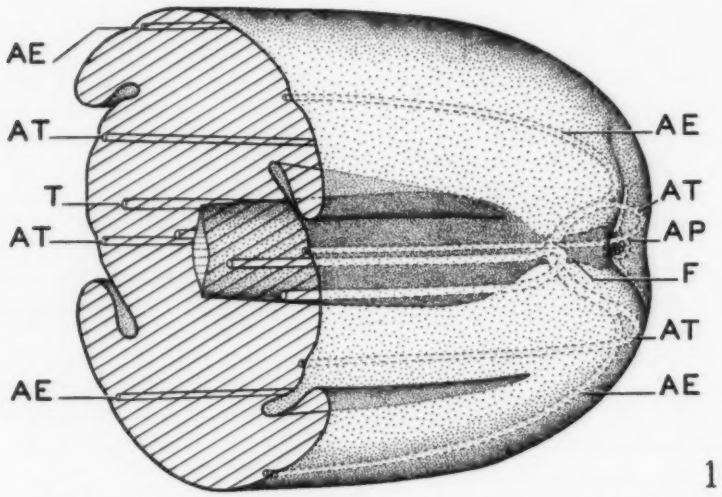


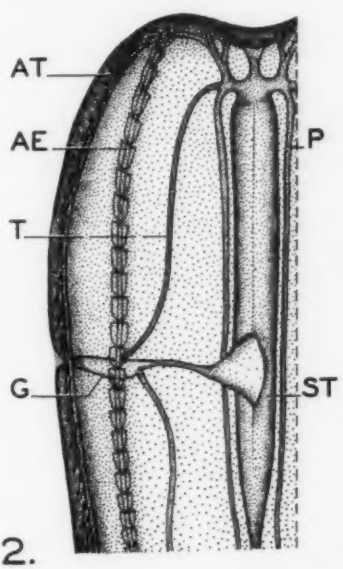
Fig. 1. This is a diagram representing the shape and symmetry of the body of *Mnemiopsis*. The plates and some of the smaller canals are not shown.

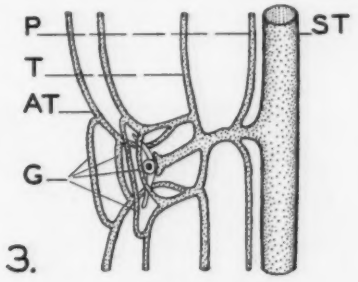
AE, adesophageal row; *AP*, apical organ; *AT*, adtentacular row; *F*, infundibulum; *T*, tentacular canal. The stomodeum is shown in the center of the drawing with the paragastic canals lying parallel to and near it.

while others had removed from them the part of the body apical to the bases of the auricles. These experiments were done in accordance with an objective somewhat different from the one included in this report. The details of these experiments will be reported later. However, as the pieces of the animal were passing through the phases of regeneration several results were pertinent to the problem considered herein.

The oral pieces usually regenerated the lost portions of the body perfectly in all details. By this regeneration the regular connections of canals and rows were soon established and the single, apical organ was

formed (Figs. 9, 15 and 17). During regeneration the two adjacent adtentacular rows and a single adesophageal row on either side of these connected to each other. As this was taking place the tip of the stomodeum formed a single bulb (Fig. 18), which immediately divided to form two bulbs (Fig. 4), and each of these became connected with a set of





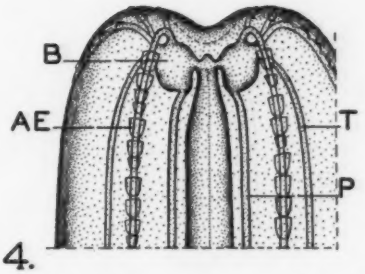


FIG. 2. An adesophageal view of *Mnemiopsis* showing a stomodeal graft after the second day.

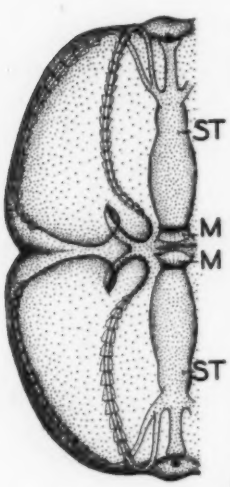
FIG. 3. This drawing shows an apical organ grafted to the adtentacular region and about mid-way between the ends of the body. The drawing was made on the fourth day after the operation.

FIG. 4. This shows the usual features at the apical end of the body during regeneration following the removal of an apical part of the animal. The two bulbs can be seen lying at the upper end of the stomodeum. This drawing was made on the fourth day.

AE, adesophageal row; *AT*, adtentacular row; *B*, stomodeal bulb; *G*, graft; *P*, paragastric canal; *ST*, stomodeum; *T*, tentacular canal.

four canals of the plate rows. The two bulbs became smaller until they assumed the size of a tube or canal and became the radial canals. At about this time also a single, apical organ formed at the tip of the stomodeum between the two bulbs. The apical plate of this organ was regularly laid down on a line between each of the two adjacent pairs of adesophageal rows (Fig. 9).

Certain irregularities were observed on the regenerating oral pieces following the cutting at each of the four levels. These consisted of a failure of the eight rows to connect as a unit resulting in the formation of two apical organs (Figs. 7 and 19), a failure of the usual row connections with or without an apical organ (Figs. 10, 14 and 16), and a splitting of the tip of the stomodeum resulting in a separation of the opposing pairs of adesophageal rows (Fig. 8). Here a single apical organ regenerated between each pair of rows and the adtentacular rows



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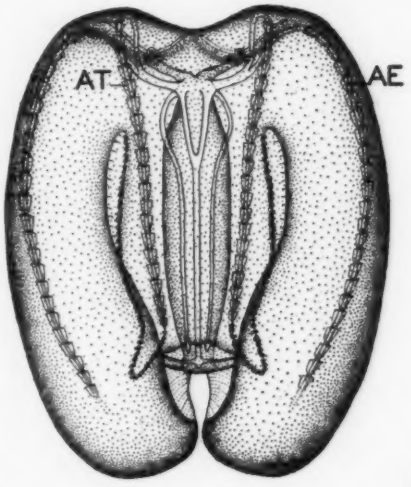

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FIG. 5. This shows the result of grafting two apical pieces together. Each piece represents less than half of an animal. The paragastric and tentacular canals are not shown. This drawing was made on the sixth day.

FIG. 6. This shows the result of grafting together two equal and longitudinal pieces of different animals. Each piece contained slightly more than one half of the body. The drawing was made on the third day.

AE, adesophageal row; *AT*, adtentacular row; *M*, mouth; *ST*, stomodeum.

did not regenerate. Although a few of these irregularities occurred on the oral piece following the cutting at each of the four levels of the body, a majority were formed by the oral pieces produced by a cross cut at the bases of the auricles (Fig. 11). The two regenerated apical organs of a single, oral piece were always at first oriented at right angles to the regular one (Figs. 7 and 8). Within a few days, however, these organs began to turn and finally they assumed the regular direction of orientation.

Experimental Graftings

Several types of graftings were done and since they all have bearing on the problem of regulation and reorganization they are listed and described here under the one heading. A few of these experiments were of an exploratory nature but their results were definite enough to make them significant. Each type of grafting is described here in a single paragraph.

1. Four mid-pieces of separate animals were grafted together with their regular orientation maintained, and the apical and oral ends of a fifth animal were grafted to the apical and oral regions respectively of the fused mid-pieces. By this method of grafting, an animal consisting of the mid-pieces of four animals with the apical and oral ends of a fifth animal was formed. All of the pieces fused at the cut regions and all canals and the stomodeum of each fused in the regular manner. Even the coördination of the plates as well as the feeding reactions at the one mouth were similar to these processes of a normal animal.

2. The apical end of an animal was grafted to the mid-adesophageal surface of another animal. This end fused to the host successfully. Soon after the fusion at the edge of the graft was complete a mouth broke through at one adtentacular region. The canals and rows of the host and the graft fused with each other and the plate movement continued in the regular manner of coördination and direction in each portion of the experimental animal.

3. Two large portions obtained by cutting two animals lengthwise at a slight angle and to one side of the stomodeum were grafted to each other (Figs. 12 and 13). In some the two large portions of animals of an equal size were fused together (Fig. 13) while in other experiments the portions were from animals of unequal size (Fig. 12). There was no apparent difference in the results of the two types of experiments. In all cases the two pieces fused within three days and the corresponding rows fused with each other. This fusion was near the ends of the rows and since two adjacent rows on a normal animal connect to each other there was no irregularity in this fusion. The plate coördination in each piece was quite regular and independent of the other piece.

4. Two pieces were obtained by making a longitudinal cut near the stomodeum and parallel to it through the entire length of the animal. The larger of these two pieces was grafted to another one of similar size and origin. In this graft the orientation of both pieces was unchanged. Fusion took place within three days and the graft continued to live as a single animal. After the operation this organism possessed two apical organs, a single stomodeum, a double number of tentacular

canals, a bifurcated paragastric canal, and a single mouth (Fig. 6). The behavior of this animal was similar to that of any normal one.

5. The two small portions of the animals which were cut in the experiments described in the section above were grafted together. Fusion took place and within six days a single apical organ regenerated and the fused pieces formed a normal animal.

6. The apical pieces of two animals were grafted together at their oral regions so as to form an animal having two opposing apical ends. These two pieces fused and healed very quickly. Although fusion took place, each piece maintained its identity by forming a mouth, by coordinating its own plate movement, and by regenerating auricles and lobes (Fig. 5).

7. Two halves obtained by cutting animals longitudinally through the adtentacular plane were grafted with their orientation reversed. The oral end of one piece was in contact with the apical end of the other piece. Fusion took place immediately and within six days the two halves began to rotate on each other, bringing the similar ends of each piece near each other. This rotation was not carried very far, however, before the specimens died.

8. The apical organ of one animal was grafted to the surface of another animal at various levels of the body. The graft contained some of the infundibulum and the bases of the adradial canals. Fusion took place immediately and the short pieces of the adradial canals connected to each other at first and later they connected to the adtentacular canals

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EXPLANATION OF FIGURES 7–11

FIG. 7. This shows the regenerating end of an oral piece. The oral piece was obtained by making a cross cut through the body at the level of the auricles. Drawn on the fourth day.

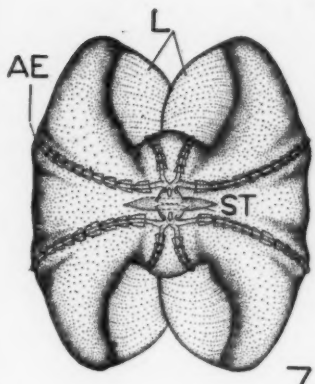
FIG. 8. A drawing of a regenerating oral piece obtained as in Fig. 7. Here the two sets of adesophageal canals are connected to the stomodeum by a separate infundibulum. The adtentacular canals failed to regenerate. Drawn on the fourth day.

FIG. 9. This shows the regular method of regeneration following the removal of an apical piece. This specimen had been cut across at the level of the auricles. The eight rows are formed and the single apical organ is shown in its regular orientation. Drawn on the fourth day.

FIG. 10. This specimen was obtained as in Fig. 9. The result of regeneration failed to bring the canals and rows to the regular type of connection. Drawn on the fourth day.

FIG. 11. This is a diagram to show the distribution of the canals and other organs in an oral piece obtained by cutting the animal across at the bases of the auricles.

AE, adesophageal rows; *F*, infundibulum; *L*, lobe; *P*, paragastric canal; *ST*, stomodeum; *T*, tentacular canal.



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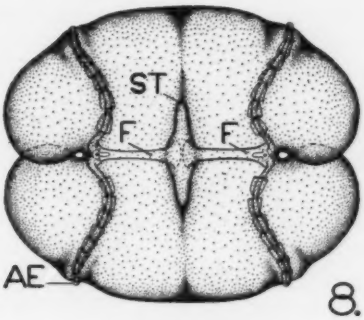

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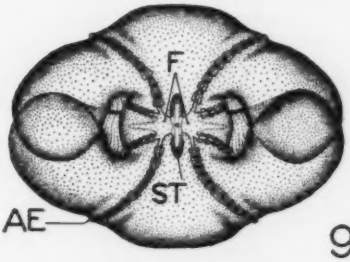

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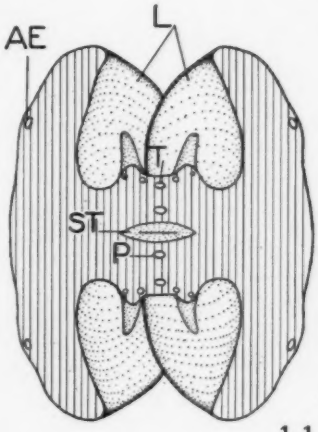

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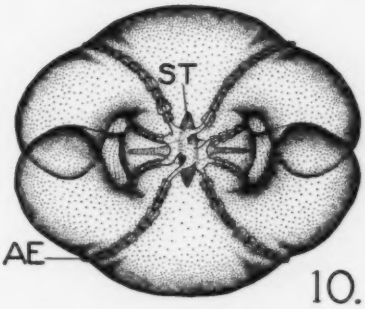

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of the host (Fig. 3). The graft always became oriented in a regular manner after its canals joined those of the host regardless of its previous orientation. Later the graft joined the stomodeum of the host (Fig. 3).

9. A small portion of the mid-region of the stomodeum of an animal was grafted to the surface of another animal between its adtentacular rows. Very soon after the healing was complete the stomodeum of the host curved out toward the graft. This bending out toward the graft took place before there was any mechanical connection between the graft

and the stomodeum. Eventually the graft and the stomodeum fused and in addition usually the tentacular canal of the host became connected to the graft (Fig. 2). The graft formed an opening at the surface of the host and thus provided an additional mouth.

Discussion

In considering the problem of symmetry in *Mnemiopsis* it is natural to observe first the anatomy of this animal. The organs are equally distributed along the opposite sides of the main, central, longitudinal axis of its body (Figs. 1 and 11). There are four plate rows, two auricles, one paragastric canal, one tentacular canal, and one lobe on each side of the stomodeum. The apical organ and the mouth are at the two poles of the stomodeum and they lie in a plane at right angles to it. Then as far as the organs, with the exception of the apical organ and the mouth, are concerned they are distributed in the body bilaterally about the stomodeum. Therefore this distribution of organs indicates a bilateral symmetry without a dorso-ventrality.

Does *Mnemiopsis* have any features other than its anatomy which might give some information about its symmetry? In answering this question I shall consider certain observations made on experiments in this animal. The outstanding feature of this animal during regeneration

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Explanation of Plate I

Fig. 12. This shows two longitudinal pieces from animals of different sizes fused together. This fusion was complete by the end of four days.

Fig. 13. This is a photograph of the fused two longitudinal pieces from animals of equal sizes. The fusion was complete by the end of the fourth day.

Fig. 14. An apical view of an oral piece obtained by cutting an animal across through its infundibulum. This shows the irregular row connection and two apical organs have regenerated. Photographed on the third day.

Fig. 15. An apical view of an oral piece obtained as shown in Fig. 14. This shows the regular row connection and but a single apical organ has regenerated. Photographed on the third day.

Fig. 16. An apical view of an oral piece obtained by cutting an animal across at the auricular region. This shows the irregular row connection and two apical organs have regenerated. Photographed on the third day.

Fig. 17. An apical view of an oral piece obtained as is shown in Fig. 16. This shows the usual row connection with but one apical organ regenerated. Photographed on the third day.

Fig. 18. This shows the formation of a single bulb at the apical end of the stomodeum. This follows the cutting off of the apical portion of the animal. The rows which are not continuous at the top of the photograph were broken during the handling of the specimen.

Fig. 19. An apical view of an oral piece obtained by making a cross cut at the region of the auricles. This shows the usual connections of the rows and two apical organs have been formed during regeneration. Photographed on the third day.

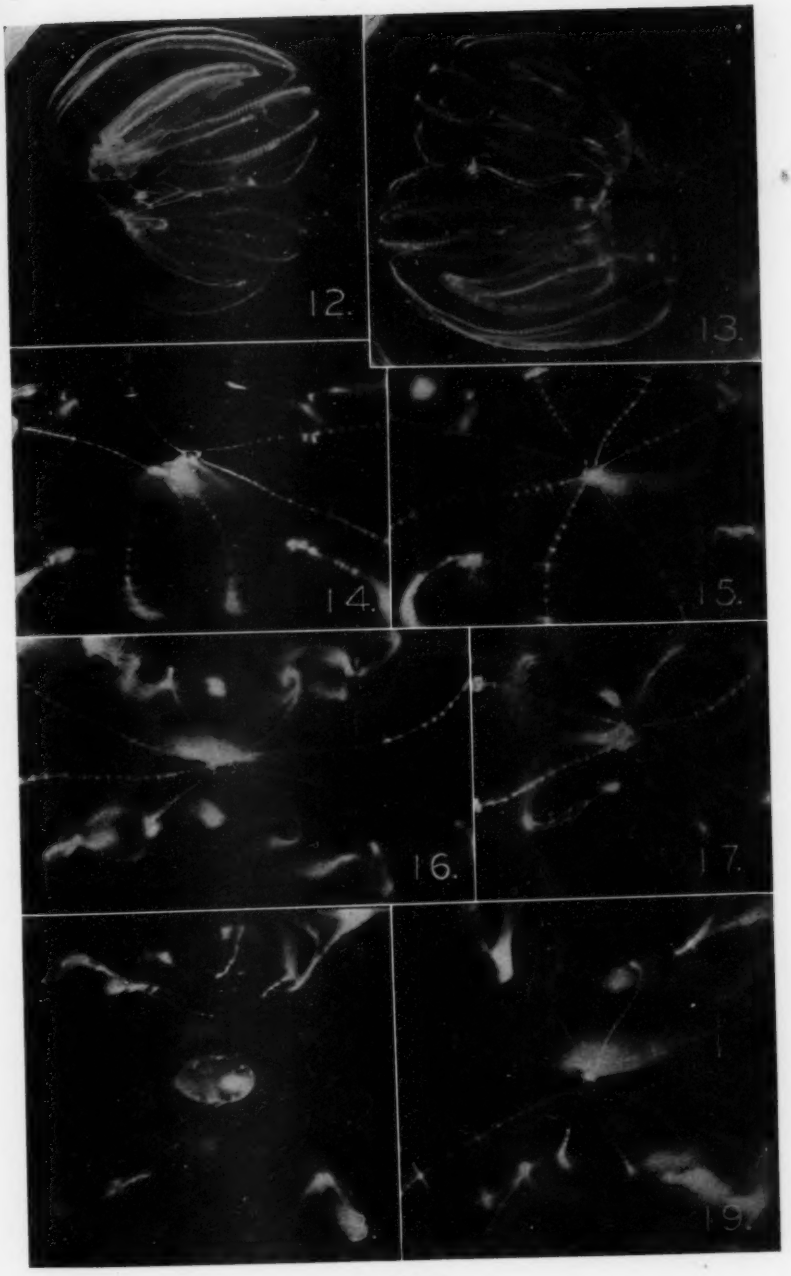

PLATE I

is its ability to regulate its own reorganization following any type of disturbance to its body that I have made. This power to regulate has been remarkably demonstrated during the process of regeneration. When this animal has been cut into halves, thirds, or fourths either by cross cuts or by longitudinal cuts the removed parts were regenerated perfectly with but few exceptions (Coonfield, 1936*a*). Following the cutting of its body across at any level above the mouth the procedure of regeneration showed a bilateral arrangement with but few exceptions. The apical end of the stomodeum formed a bulb (Fig. 18), then this bulb split in two (Fig. 4). Later these bulbs thinned out and formed the two radial canals. Both the tentacular and the paragastric canals established their regular connections with these two bulbs (Fig. 4) and as these bulbs disappeared as such these canals became connected to the base of the infundibulum (Fig. 2). In the meantime the apical end of the stomodeum became elongated and formed the infundibulum with a single apical organ formed on the end of this structure. During the regeneration of the apical organ the plate rows were regular in their reformation. Two adjacent adtentacular rows and a single adesophageal row on either side of them became connected to each other at first (Fig. 9). Then as the bulbs became the radial canals the plate rows established their usual connection to the infundibulum by way of the radial canals. The regular organization was complete after the apical organ had regenerated.

How can the irregularities in regeneration be accounted for even though they are relatively few in number? These irregularities have appeared following the cutting of the animal into halves, thirds, and fourths (Coonfield, 1936*a*). A very few of the regenerating pieces failed to reform the lost plate rows. A few irregularities are given in the present report. These are concerned with the failure of the rows to become connected in the regular manner (Figs. 10, 14 and 16), or with the regeneration of two apical organs instead of a single one (Figs. 7, 8, 14, 16 and 19), or with a considerable splitting of the stomodeum to form two infundibula (Fig. 8). Even though I cannot offer a satisfactory reason for the appearance of these irregularities I believe they are significant in supporting the view of bilateral symmetry. For in all except three or four that failed to reorganize the plate rows in the regular manner the reforming organs were distributed according to bilaterality. So then the regulation of the reforming organs in the body of *Mnemiopsis* whether they proceeded in the regular manner or exhibited some irregularities supports the view of bilateral symmetry.

Information having a direct bearing on the problem of symmetry and regulation in addition to that shown by a study of the anatomy of *Mne-*

miopsis and observations on regeneration in this animal was obtained by performing experiments involving grafts. This animal showed a remarkable ability to regulate itself both according to structure and physiology in response to this type of experiment. The ability of this animal to organize the grafts in experiments wherein a whole body was formed by fusing the mid-pieces of four animals with the apical and oral pieces of a fifth animal, the noninterruption of the regular activities of both the graft and the host following the fusing of an apical piece at right angles to the surface of the host, and also wherein a large portion of two animals were fused together (Figs. 12 and 13) show the ability of this animal to regulate itself. The reorganization following the fusion of two longitudinal parts of the body (Fig. 6) shows that the animal almost succeeded in organizing a normal body. This opinion is confirmed by noting that the two parts of the stomodeum fused as one and with but one mouth, the two paragastric canals on each side of the stomodeum united all along except near the infundibulum, and the actions of this animal were similar to those of a normal one. In the experiments whereby two apical pieces were fused to each other at their oral regions the ability of each piece to regulate itself although being fused to another similar one was definitely shown.

Further evidence supporting the view of regulation in a physiological manner was obtained by observing polarity in *Mnemiopsis* following experiments involving grafts. Polarity has been observed previously in this animal (Coonfield, 1934 and 1936*b*). The data contained in the present report show that when two halves of an animal were grafted together with their orientation reversed each piece attempted to rotate so as to bring the two similar regions together. Also when an apical organ or a piece of the stomodeum was grafted to the surface of an animal these grafts always became oriented and established connections to the plate rows or the other canals, and to the stomodeum (Fig. 3). It is interesting to note that the apical organ became oriented only after connections to the canals had been established and both the canals and the stomodeum were attracted to the stomodeal grafts immediately after each had become healed in the body of the host. The apical grafts persisted long enough to show that their form and function were unchanged by their new location. Also the stomodeal grafts which were taken from the side of the stomodeum not only continued to function as a stomodeum but in addition each formed a mouth. In summing up the data obtained from observing the various types of grafts which were carried out on *Mnemiopsis,* it seems to me that these data show that regulation is demonstrated by them in this animal. Since regulation in this

animal can be associated with symmetry, it seems that these experiments support the view of bilaterality without dorso-ventrality.

Conclusions

1. *Mnemiopsis* possesses a bilateral symmetry without a dorso-ventrality. This is shown by the anatomy of this animal and by its method of regulation following a disturbance to its body.

2. Symmetry and regulation are two closely associated features of *Mnemiopsis.*

3. Regulation is demonstrated in *Mnemiopsis* by the following results.

a. Reorganization during regeneration which follows the cutting of its body.

b. Reorganization during the fusion, orientation, and connections of grafts.

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ON THE SIGNIFICANCE OF THE POLAR SPOT IN RIPE UNFERTILIZED AND IN FERTILIZED ASCIDIAN EGGS

A. DALCQ (Brussels) AND G. VANDEBROEK (Ghent)

Cohen and Berrill (1936) have given an interesting account of observations on eggs of *Ascidiella aspersa* and *Phallusia mamillata*. After the jelly was digested by the gastric juice of the crab *Munida*, the eggs were stained *in toto* with a vital dye (particularly Nile-blue sulfate) and subsequent events studied with special reference to changes in the form of the germ and in the appearance of the vital dye within the egg. These are described in the ripe egg, from the time of fertilization until the formation of the polar bodies, and during cleavage, gastrulation, and formation of the tadpole.

In the ripe unfertilized egg they have observed the "polar pit" ("tache polaire" of *Ascidiella*—Dalcq, 1932) though they question whether this cortical differentiation marks the position of the maturation spindle and identifies the location of the animal pole. Their doubt is not based on continuous observation of the region of the polar pit from which the dye entirely disappears before the elevation of the polar body, nor have they been able to determine directly whether the polar body is actually formed in the polar pit or not. They are influenced, however, by certain cases in which foreign particles adhere to the cortical layer of naked eggs in the vicinity of the polar pit. On measuring the distance between such a particle and the pit, and, later, between the same particle and the polar body, they found considerable variation in the distances. In two cases, drawings of which are given in their paper, the distance was increased; the same was true of "several" other eggs. In one case the distance did not change. While Cohen and Berrill consider the possibility of a displacement of the marking particle by some movement of the cortical layer, they discard such an explanation on the following grounds. During the maturation period the egg shows, grossly, only a flattening and an elongation, while the deformation during cleavage, though much greater, is unaccompanied by any change in the distance between the polar body and the adhering particle. Hence they do not believe it possible that the earlier deformations of the egg could be responsible for the displacement of the small body fixed to the cortex. They feel justified in stating, therefore: "Since Dalcq accepted the site of the polar pit as being identical with that of the polar body and

used it to orient the eggs for cutting, it follows that some doubt is thrown on the validity of his conclusions regarding the localization of presumptive germinal regions in the fertilized egg of *Ascidiella aspersa*, although, of course, not on the existence of such presumptive regions" (p. 84). They do not state what, in their opinion, the significance of the polar pit is. On the basis of experiments cited below we have good reason to believe that their conclusion is not justified.

It is unfortunate that Cohen and Berrill have discarded the possible explanation of the behavior of the attached particles in their experiments on the basis of cortical movements. Such movements are common in eggs and may be easily identified by means of definitely localized vitally stained areas which may be followed through ensuing stages. This method was used by one of us (G. V.) at Roscoff [1] during the summer of 1935 with outstanding success. We shall mention here only the results necessary to dispel the doubt thrown by Cohen and Berrill on the significance of the polar "pit," perhaps better called the polar "spot." In addition, we shall describe briefly an anomaly observed by the senior author (A. D.) which gives further proof of the existence of a maturation spindle immediately under the polar spot.

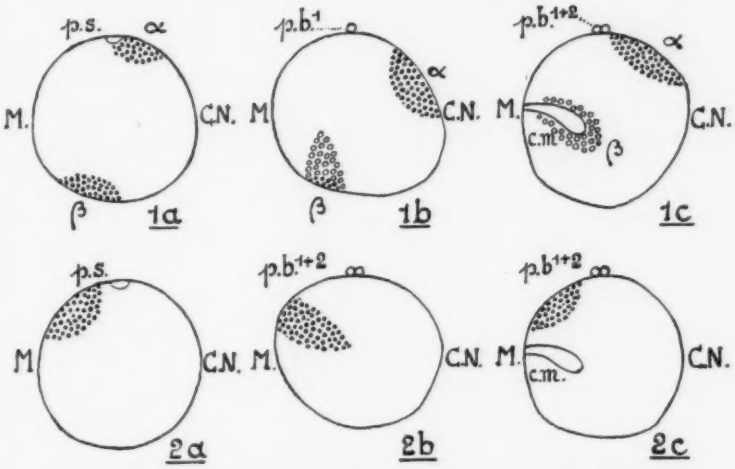
The ripe egg of *Ascidiella* [2] contains the first maturation spindle, as stated by Cohen and Berrill, and not the second, as previously stated by Dalcq on the basis of the examination of unfertilized eggs and the appearance of the spindle in sectioned preparations. On continuous observation two polar bodies may be seen to be successively extruded by the egg after fertilization. The jelly of the egg was removed with the aid of mounted needles (watchmakers' forceps can also be used), some dozens of naked eggs easily being prepared in but a few minutes. The method is not tedious.

In the discussion of merogony experiments Dalcq has already called attention to the fact that after insemination of naked eggs, the penetration of the spermatozoön into the egg may be delayed by as much as

[1] We wish to express our gratitude to Professor Dr. Ch. Pérez and Dr. G. Teissier for the excellent working opportunities accorded both of us during several stays at the "Laboratoire Lacaze-Duthiers."

[2] Because of some confusion as to the identification of the species used, it is necessary to state that our experiments were made on the *Ascidiella* which is found in abundance in the great pool of the Roscoff Laboratory, and which was used by Dalcq and by Tung in 1931 and 1932, and called by them *Ascidiella aspersa*. Berrill (1928) suggests that, because of the small size of the animal and the fact that the eggs do not float, the Roscoff species should be called *Ascidiella scabra*, the name of *Ascidiella aspersa* being reserved for the great *Ascidiella*, with floating eggs, common in Plymouth (and in the vicinity of Boulogne). Dalcq and Tung, following this suggestion, used the name *Ascidiella scabra* in their subsequent publications.

half an hour. Vandebroek has more recently discovered, in the course of experiments which will be reported in detail elsewhere, that the moment of sperm penetration can be recognized by a series of characteristic deformations of the egg which persist for about two minutes. The egg then returns to the spherical condition until the time of first polar body formation. As is noted below, the first and second polar bodies are formed 6 and 20 minutes respectively after the penetration of the spermatozoön. The actual times vary with temperature but the above are those observed in the present experiments. In dealing with eggs in



FIGS. 1 AND 2. Three views of eggs subjected to local vital staining: (*a*) ripe egg; (*b*) after the formation of the first polocyte; (*c*) during the formation of the yellow crescent and hyaline plasma zone. Colored territories stippled in deep black, when superficial invaginated parts (Figs. 1*b* and *c*) marked with ooo; *p.s.*: polar spot; pb^1: first polocyte; pb^{1+2}: the two polocytes; *M.*: mesoblastic side; *C.N.*: chordoneural side; *c.m.*: mesoblastic region (yellow crescent). The two eggs are viewed from their left sides.

the brief period preceding maturation it is especially important to recognize the delay mentioned above and to identify the moment of sperm penetration. This, unfortunately, Cohen and Berrill have failed to do.

In the experiments reported below, localized areas of denuded ripe unfertilized eggs were vitally stained with the dye 'Brillant Cresyl.' The stained areas have a diameter of from 50 to 75 μ (ca) and persist, perfectly localized, during the hours immediately following fertilization. If the egg is not fertilized the dye rapidly diffuses. Three experiments which demonstrate the significance of the polar spot will be described.

In the first case (Figs. 1*a*, 1*b*, and 1*c*) one mark (α) was made in the immediate vicinity of the polar spot and another (β) on the vegetative pole (Fig. 1*a*). In the second case one mark only was made, near the polar spot (Fig. 2*a*). In the third egg the mark was located a short distance from the pole, between the polar spot and the equator (Figs. 3*a* and 3*a'*). Tracing the fate of mark α of the first egg, we see that two minutes after fertilization the angular distance between the polar spot and the adjacent border of the colored region is increased. Comparing this with the similar behavior in the other eggs, it may be noted that all of the marked areas on the animal hemisphere have in-

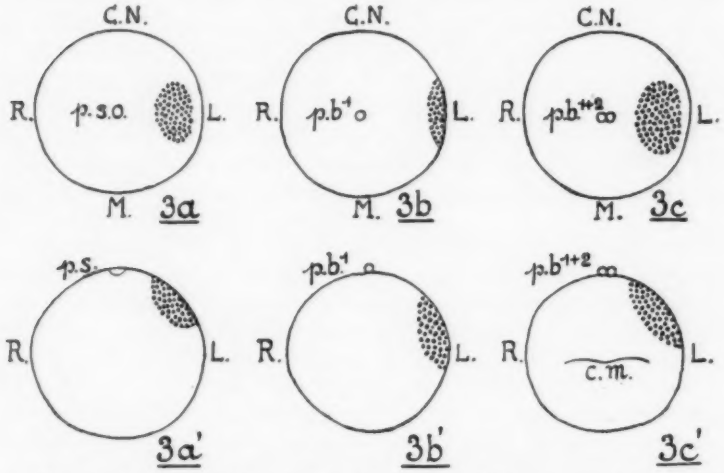


FIG. 3. An egg with lateral coloration of the animal pole. *a, b, c:* viewed from the animal pole; *a', b', c':* viewed from the mesoblastic side. Other abbreviations as in Figs. 1 and 2.

creased their distance from the polar end by about 30° (Figs. 1*b*, 2*b*, 3*b*, and 3*b'*). They maintain these positions in spite of the considerable deformations which the egg undergoes during the period of expulsion of the two polar bodies. In the case illustrated by Fig. 1, the presence of the mark enables us to determine that the first polar body is actually formed in the polar spot about 6 minutes after fertilization. The second polar body is formed next to the first about 14 minutes later (Figs. 1*b* and 1*c*). It appears, therefore, that the polar spot is also the maturation site.

In the post-maturation phases during which the female pronucleus migrates toward the center of the egg to meet the male pronucleus and

the yellow crescent is gradually formed, the behavior of the marks in the vegetative pole is very interesting. This is to be described in detail elsewhere (by G. V.) but is suggested by the mark β of case 1. In the present consideration we are directly concerned with marks on the animal half of the egg and these consistently show a movement toward the animal pole which results in their return to their original positions (Figs. 1*c*, 2*c*, 3*c*, and 3*c'*). They show no further change during the cleavages which follow.

The appearance of the yellow crescent and the adjacent hyaline protoplasm which contains the fusion nucleus allows one to determine definitely the orientation of the egg. Using these landmarks, it was found that in the egg of Fig. 1, the mark α was in the plane of symmetry, just above the chordoneural material, while the mark β lay under the future yellow crescent, i.e. on the mesoblastic side of the germ. In the second egg the mark had been put between the polar spot and the presumptive mesoblastic crescent, nearly in the plane of symmetry. In the third case, on the other hand, the mark was located in a region to one side, in the left half of the germ. In these three typical experiments, therefore, the chief regions around the polar spot have also been explored.

To summarize briefly, it has been found that, after fertilization, the cortical layer of the ascidian egg undergoes definite displacements. During the initial period of active deformations, the cortical layer surrounding the animal pole moves toward the equator. Later, when the conjugation of the pronuclei takes place, the same material is again shifted nearer to the animal pole. In conclusion, it may be stated without the least hesitation that the polar spot indicates the site of formation of the polar bodies, and is the result of the presence of the first maturation spindle just below the cortex.

In view of these conclusions the question now arises as to how the data of Cohen and Berrill are to be interpreted. It seems clear that the variations they record in the distance between the adhering particle and the animal pole result from the activity of the cortical film; the cases in which the distance did not change may be explained on the assumption that the eggs were first observed somewhat too late. This explanation is not entirely satisfactory, however, for they appear to have observed some of the eggs continuously and yet fail to record any secondary shift of the materials towards the animal pole. In this connection we should like to make several suggestions inasmuch as it must be admitted that observations made by means of adhering particles are less reliable than those based on local vital staining of parts of the egg itself. The possibility of a displacement of the particle during the later deformations of

the egg should be examined. It is possible that the particle might eventually penetrate the cortical film and adhere directly to the underlying granular cytoplasm.

For the latter form, the morphological value of the polar spot is now established beyond any doubt and it would appear that Dalcq was justified in basing his merogony experiments on this indication of the animal-vegetative axis of the egg. Sectioning of the ripe ascidian egg in various planes relative to this primary axis and subsequent fertilization

FIG. 4. Reconstruction of a fixed abnormal *Ascidiella* egg. Fixation, Allen; Sections, 6 μ; Staining, Hollande. Perspective view at 45° (method of Lison). *a, b, c:* the three maturation spindles. The eosinophil subcortical material is stippled.

of both pieces seems to be at present the sole method of analysing the distribution of the potencies at that stage. Further experiments have been carried on by the senior author during recent years and a short account of the results has been presented (Dalcq, 1935). This has led to a further cytological study of the structure of the ripe egg, and to the discovery of a preëxisting bilateral symmetry indicated by the arrangement of eosinophil subcortical granules (the material of the future yellow crescent) in the form of a large crescent which is more developed on one side of the primary axis. This important feature of the organization of the ripe *Ascidiella* egg together with the variations in the structure of merogonic twin embryos, whose anatomy has been thor-

oughly studied in a quantitative way, with several graphic reconstructions, will be fully described in another place.

In relation to the problem of the significance of the polar spot considered here, it seems important to draw attention to an anomaly which has to be borne in mind when performing merogony experiments. Among numerous sections of ripe eggs, the senior author has observed, four or five times, eggs which appear to possess two polar spots. Such eggs were at first discarded without further consideration, but later, a similar case was fixed and sectioned. The slide shows that the egg contains no less than three maturation spindles. In Fig. 4 may be seen a graphical reconstruction of the egg by Lison's method (1936). The three spindles lie in the region free from the eosinophil granules which nearly cover the vegetative hemisphere. The three spindles are small, of approximately equal size, and each supports some chromosomes. Owing to the direction of the plane of sectioning, which was more or less parallel to the primary axis of the egg, two of the spindles (*a* and *b*) lie under the part of the cortex toward the observer of the drawing; the third *c*, is situated on the opposite side, not so far from *b* as it appears to be on the drawing.

The infrequent occurrence of such eggs is of some importance to the investigator who is performing merogony experiments and throws some light on the significance of the polar spot. It is clear that the existence of a secondary spot corresponds here to a supplementary spindle. Why two and not three spots were seen may be explained in either of two ways; either one of the spots escaped the eye of the observer, or one of the spindles did not adhere sufficiently to the cortex to be seen *in vivo*. No positive information has been obtained concerning the origin of such anomalies. It seems probable that the spindle material became divided when the rupture of the germinal vesicle took place.

Summary

Ripe *Ascidiella* eggs have been subjected to local vital staining, chiefly in the regions surrounding the animal pole. Fertilization is immediately followed by a shift of the cortical layer towards the equator; when the yellow crescent appears, the material returns to its original position. The continuous observation of the ripe and the fertilized egg shows, contra Cohen and Berrill, that the polar spot marks the place where polocytes will be extruded.

In addition, attention is called to cases where there are two or three maturation spindles in the same egg.

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REPRODUCTIVE SYSTEM AND COPULATION IN AMPHISCOLOPS LANGERHANSI (TURBELLARIA ACOELA)

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The Turbellaria Acoela constitute one of the most interesting invertebrate groups because they are the lowest of the bilateral animals (if the Mesozoa be excepted). Their simplicity of structure is in all probability primitive and not the consequence of degradation. Among their primitive characters are the absence of a definite digestive entoderm, the presence of a statocyst, the netlike nervous system with radially arranged principal nerve strands, the scattered sex cells, the absence of female ducts, and the simple construction of the copulatory mechanism. In the Acoela the entoderm has apparently not yet differentiated from the general mesentodermal cells. In the most primitive genus, *Nemertoderma,* a definite brain is lacking, the nervous system consists of a subepidermal plexus, and the reproductive system is limited to the sex cells, as ducts and copulatory apparatus are entirely wanting (Steinböck, 1931). The extremely complicated reproductive system characteristic of the Platyhelminthes has evidently arisen within the phylum itself, starting from conditions in the Acoela, where this system may not have advanced beyond the stage seen in cœlenterates, as in *Nemertoderma.* The first step in the development of a reproductive system among the Acoela consists in the formation of more or less definite paired channels in the parenchyma for the transport of the sperm. These somewhat vague vasa deferentia unite to a common terminal duct in whose wall a penis gradually evolves, first as a muscular thickening of the wall, then in most Acoela as a conical penis composed of parenchyma, muscle fibers, and in some cases gland cells also. Female ducts are absent throughout the group but in the majority there is a seminal receptacle, the seminal bursa, in the form of a rounded parenchymatous organ which may or may not be connected to the exterior either by its own pore or by way of the male duct and pore. This seminal bursa has no direct connection with the eggs but possesses a variable number of projecting exits, composed of a hard material arranged in rings. These exits were termed "mouthpieces" by Mark (1892) and his name has been adopted by German workers in the form Mundstück, but they so

much suggest the nozzle of a hose that I shall call them nozzles. In some Acoela there are numerous seminal bursæ each with one nozzle.

It is thus evident that most of the Acoela possess a copulatory mechanism in the form of a protrusible penis papilla and a seminal receptacle but to my knowledge copulation has never been recorded for the group. The only account in the literature which I have been able to find is that of Gardiner (1898), for *Polychœrus caudatus*. Gardiner noticed that restless individuals often mounted on quiescent ones and remained in this position for a brief period. Sections of the quiescent worms showed sperm on the surface and in the parenchyma and Gardiner therefore concluded that in this species fertilization occurs by means of hypodermic impregnation. While hypodermic impregnation is probably of wide occurrence in the Acoela it is to be presumed that a true copulation also takes place since the necessary mechanism is present, as von Graff has pointed out (1908).

During a stay at the Bermuda Biological Station in the summer of 1935 I had ample opportunity to witness the breeding habits of an acoel present in the aquaria there. After careful study of living intact and pressed specimens and serial sections, I have identified this animal as *Amphiscolops langerhansi* (Graff) 1882. Specimens agree in all details with von Graff's 1904 description. This species has flourished and bred for some time in the aquaria at the Bermuda station but its source is unknown. Dr. Wheeler, the Director, informed me that the animal had not been found in nature at Bermuda although I collected a very closely related form from the sea-weeds near the station. The species has apparently been introduced into the aquaria from some outside source. It is recorded by von Graff from Madeira, the Canary Islands, and the Mediterranean.

Amphiscolops langerhansi (Fig. 1) is a small worm, reaching 4–5 mm. in length, and of elongated form with a bilobed posterior end. Near the anterior end is a conspicuous statocyst and two small eyes. The brown color is due to numerous zoöxanthellæ situated in the peripheral part of the parenchyma. A study of their rôle in the life of the animal has recently been made by M. F. Welsh (1936). In addition there occur just under the epidermis areas formed of an apparently crystalline material, called concrement crystals by von Graff; they are dark by transmitted, opaque white by reflected light and confer a pattern on the animal. This pattern differs considerably in different individuals but its general arrangement is indicated in Fig. 1. The mouth is central and near the posterior end can be noticed the rounded mass of the penis.

Although the reproductive system of *A. langerhansi* has been ade-

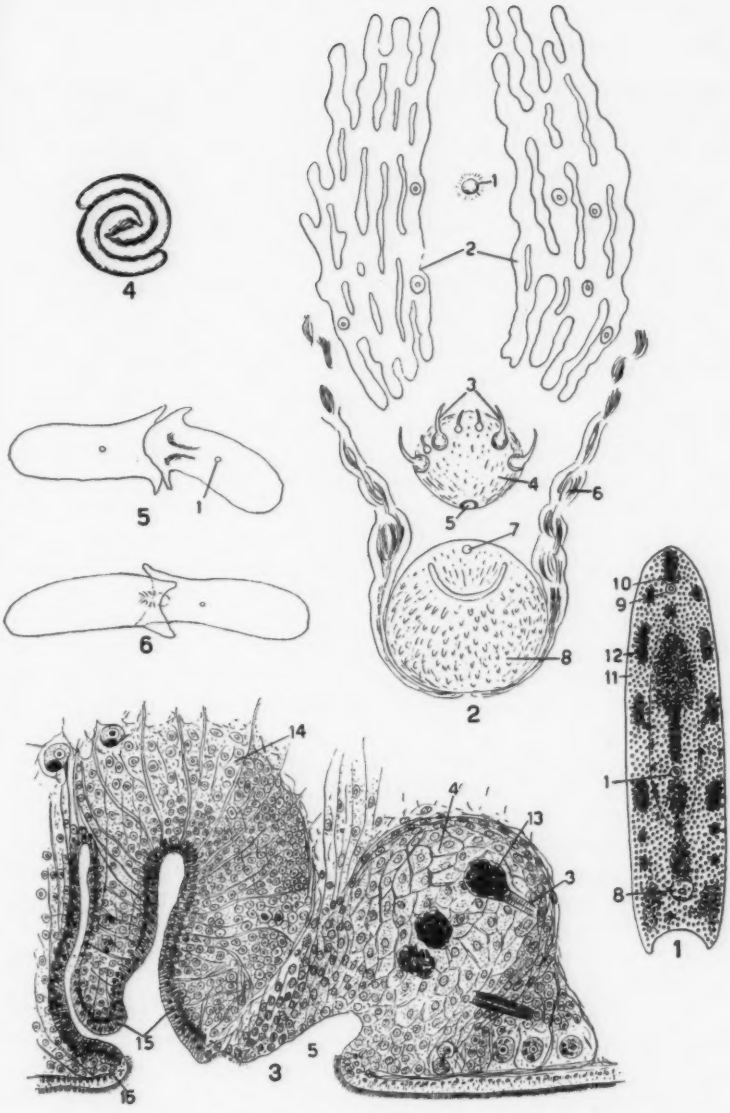

FIG. 1. *Amphiscolops langerhansi,* from life, showing the pattern of concrement granules.

FIG. 2. General view of the reproductive system in a living pressed specimen.

FIG. 3. Penis and seminal bursa in sagittal section.

FIG. 4. Beginning of copulation, rolled-up stage.

FIG. 5. Attitude during copulation.

FIG. 6. Copulating pair in motion, right-hand individual advancing and dragging left-hand one with its dorsal surface against the glass.

quately described and figured by von Graff (1904), it is necessary to review its main features in order to understand the copulatory process. Its general appearance as seen in a live worm pressed under a coverglass is shown in Fig. 2. The ovaries consist of strands of eggs in a paired lateral location. Behind and unconnected with the eggs lies the median rounded seminal bursa, or sperm receptacle. This bears several anteriorly directed nozzles. The number of these is variable and increases with age so that small ones occur along with large ones in the larger specimens. Von Graff records finding 6–11 nozzles and I have found 2–8, usually 4 large ones and one or more small ones. These nozzles consist of a hard yellow material, commonly spoken of as chitinous; but no proof exists of the occurrence of chitin in the Platyhelminthes and they are probably cuticular in nature, that is, composed of a scleroprotein allied to keratin. The nozzles are cross-striated and appear to consist of a series of superimposed rings. They are pointed towards the ovaries and serve to discharge sperm against the eggs. The end of the nozzle buried in the tissue of the bursa connects with a rounded sac filled with sperm. The bursa opens below by the female genital pore. Immediately behind the bursa lies the large rounded mass of the penis filled with gland cells. To either side a somewhat indefinite channel, the vas deferens, containing masses of sperm, approaches the penis, passes around to its posterior side and there opens into the penis base. The testes consist of cell groups scattered throughout the lateral parenchyma.

For an adequate understanding of bursa and penis, sections are necessary. This region of the animal is illustrated in Fig. 5, a median sagittal section. The seminal bursa is composed of loose reticular fibers in the meshes of which parenchyma cells occur. It has no definite cavity but there are many small spaces and several larger spaces each occupied by a dense mass of sperm. From each such sperm mass a nozzle extends anteriorly. Posteriorly and ventrally the bursa opens into the female genital pore by way of a small invagination which is not lined by a definite epithelium.

The penis considerably resembles that of triclads but is of a looser, less muscular construction. It consists of a loose mass of parenchymal and gland cells, not well differentiated from each other, through which run radiating muscle fibers. These muscle fibers join the general subepidermal muscle layers. As in triclads, the penis is divisible into an internal mass, the penis bulb, continuous with the general parenchyma, and the projecting papilla. This papilla is continuous anteriorly with the parenchyma of the seminal bursa; it is free only posteriorly, where it is separated by a deep cleft from the succeeding part of the body.

The body wall behind this cleft forms an anterior projection which unexpectedly has been found to play an important rôle in copulation. I shall therefore call it the sperm guide. Unlike the rest of the body surface, which has no definite epithelium (the epidermal cells are "eingesenkt" as the Germans say) the penis lumen and cleft separating penis papilla from the sperm guide are lined by a more or less definite epithelium. The cavity in which the penis papilla lies opens ventrally by the male pore which is so close behind the female pore that the two may be considered with von Graff to form a common shallow space.

In one aquarium at Bermuda which contained five or six hundred specimens of *Amphiscolops*, copulation was observed on numerous occasions. At all daylight hours from 6 A.M. to 5 or 6 P.M., one or two to a dozen pairs in copulation could be seen on the wall of the aquarium facing the light. Apparently no copulations occur after dark and usually the number decreased after 4 P.M. As the number of sexually ripe individuals in the tank was estimated at less than two hundred and as fifty or more copulating pairs were counted daily, it is evident than any one animal must copulate repeatedly. To test this, five copulating pairs were isolated into a finger bowl. The following day, two egg masses were found in the bowl and one pair was again in copulation.

Copulation occurs in the following manner. When two individuals wandering on the aquarium wall happen to come in contact they generally give each other quick little touches resembling nips with the anterior end. This behavior commonly occurs whether copulation ensues or not. Only the larger specimens copulate. Often a large specimen was seen attempting copulation with a smaller one which declined the invitation. If both individuals are ripe for copulation, they, following a short period of the nips just mentioned, suddenly roll up into a ball (Fig. 4), with one individual on the outside. The posterior end of this individual is so curved that its ventral surface is in contact with the ventral surface of the tail end of the other copulant. After a very short interval, less than thirty seconds, the two worms unroll and are seen to be in firm connection. The upper individual simply flattens its ventral surface against the glass and becomes quiescent. The other one is compelled to twist about until its ventral surface touches the glass. Both then become motionless with ventral surfaces against the glass and heads pointed more or less directly away from each other (Fig. 5). If disturbed they will crawl about, one animal with ventral surface against the substratum dragging the other, dorsal side next the substratum, after it (Fig. 6). The union is thus very firm, and the copulatory act is prolonged. In a number of undisturbed pairs which were timed connection lasted from forty to fifty-five minutes, usually about fifty

minutes. The animals then quickly separate, each gives a comical little shake, as if settling its viscera into place, and proceeds about its business.

The process of copulation connot be understood by a mere inspection of copulating pairs. Indeed, study of these with a high-power hand-lens was puzzling rather than informative for the location of the

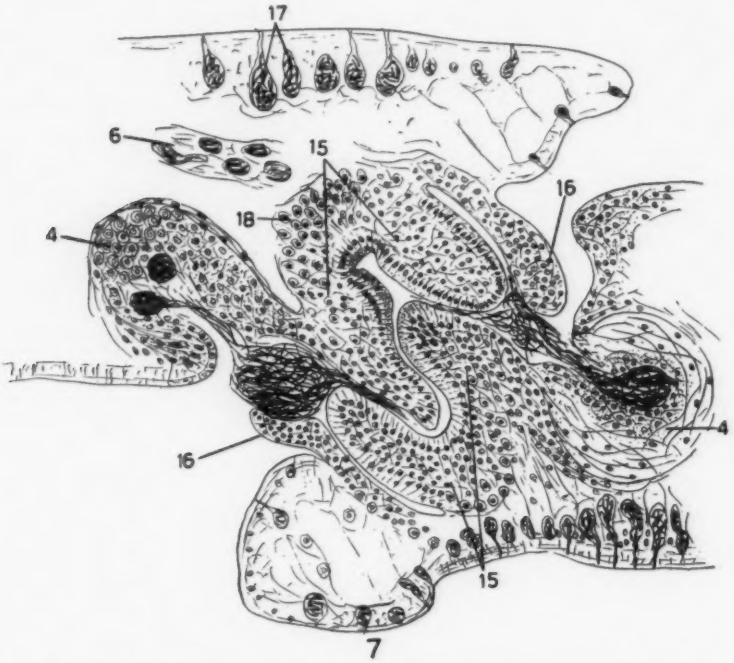

FIG. 7. Section through a copulating pair, reconstructed from several sections, showing manner of interlocking of the penes and stream of sperm, directed by the sperm guide, flowing into the seminal bursa of each copulant.

In all figures: 1. mouth, 2, ovary, 3, nozzles of seminal bursa, 4, seminal bursa, 5, female genital pore, 6, vas deferens, 7, male genital pore, 8, penis, 9, statocyst, 10, eye, 11, zoöxanthellæ, 12, area of concrement granules, 13, sperm mass in bursa, 14, penis bulb, 15, penis papilla, 16, sperm guide, 17, cutaneous glands, 18, gland cells of penis bulb.

connection indicated that the penis papilla was not inserted into the seminal bursa. The natural assumption, of course, is that the penis papilla discharges directly into the bursa and a statement to this effect is made by Bresslau (1933, p. 118). Inspection of Fig. 3 shows, however, that the seminal bursa has no definite lumen and that the female

genital pore is too small and shallow to receive the penis. Fortunately it is easily possible to fix pairs in copulation and several such have been studied in serial sections.

A sagittal section of a copulating pair is drawn in Fig. 7. It is seen that not only the penis papilla but also the sperm guide is protruded and that the penes are interlocked in a dovetailed fashion such that the lumen of each penis grasps the posterior part of the penis of the partner. This leaves the anterior part and sperm guide of each copulant directed towards the bursa of the other. The sperm issue in a stream from the penis lumen and pass over the anterior edge of the partner's penis towards the latter's bursa. They are prevented from escaping into the sea-water by the sperm guide which directs them towards the bursa. They enter the bursa and pass into the sacs connected with the nozzles. Study of serial sections of the bursæ of copulating animals has shown that the sperm easily penetrate throughout the bursa by way of spaces and so the one stream of sperm reaches all of the sperm sacs in the bursa. It is evident that some glandular secretion accompanies the sperm for the lining epithelium of the proximal part of the penis lumen contains secretion granules and a secretion staining with hæmatoxylin and thus probably albuminous in nature also is found in the penis lumen.

The copulation is thus seen to be mutual as in the Platyhelminthes in general, each worm fertilizing the other. The manner of copulation is, however, unique, consisting of an interlocking of the penes. The only comparable case that occurs to me is that of the slugs where in many species the penes twine about each other in copulation and deposit a sperm mass upon each other.

The eggs are laid at night, probably after midnight, in flat gelatinous cakes stuck to the aquarium glass or other objects. The material in which the eggs are imbedded is probably secreted by the very numerous cutaneous glands. It is evident from anatomical considerations that the eggs cannot be laid by way of the female genital pore. They probably issue through the mouth but unfortunately the process was not observed. The number of eggs in the egg mass was counted in 44 cases and found to range from 3 to 16, with the majority containing 6 to 9 eggs. The eggs hatch on the fifth day into completely formed little worms which, however, do not possess any zoöxanthellæ.

Early in the morning, about 6 A.M., the eggs laid during the night are found for the most part in 16- to 32-cell stages but a few earlier cleavages can generally be discovered. The early cleavages were watched in a number of eggs and did not seem to accord with the standard account, derived from the work of Bresslau on *Convoluta* (1909, 1933). The first cleavage was sometimes equal, sometimes unequal. A three-

cell stage was common and the four-cell stage showed no definite arrangement into two micromeres and two macromeres. The cleavage of the four-cell stage was not followed satisfactorily owing to the sudden shifts in position occurring at the moment of cleavage. The time remaining after the animals began to lay eggs was too brief to permit any extended study of the cleavage, but a number of egg masses were preserved and may be studied at some future time.

For the privileges of a stay at the Bermuda Biological Station I am greatly indebted to the President, Professor E. G. Conklin, and the Director, Dr. J. F. G. Wheeler.

Summary

In the acoelous turbellarian *Amphiscolops langerhansi,* copulation occurs by the interlocking of the penes. The sperm pass over the anterior surface of the partner's penis into the seminal bursa of the latter. The body region immediately behind the penis is also protruded in copulation and acts as a sperm guide directing the sperm into the partner's bursa and preventing their dissemination into the water.

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A NEW GENUS OF HYDROID AND ITS METHOD OF ASEXUAL REPRODUCTION

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(From the Mount Desert Island Biological Laboratory)

A tubularian hydroid (Plate I) was found in the waters of Frenchman's Bay off Salsbury Cove, Mt. Desert Island, which apparently is a hitherto undescribed genus. It is a solitary form dredged off mud bottom at about 40 to 60 feet along with *Corymorpha pendula* and *Acaulis primarius*. Many specimens were found. Its peculiar interest lies in an unusual mode of asexual reproduction.

The name *Dahlgrenella farcta*, nov. gen. et nov. sp., is proposed for this form. The generic name *Dahlgrenella* is suggested to express my indebtedness to Professor Ulric Dahlgren of Princeton University for his kind assistance and advice in this work, as well as for his method of collecting mud-living hydroids.[1] The specific name *farcta* is descriptive of the method of asexual reproduction described above, being the Latin word for "stuffed." The ovoid bodies resemble a string of sausages, the word for which in Latin is "farcimen" from the same root as *farcta*.

Doctor Willie W. Smith of New York University was particularly helpful in the preparation of the manuscript for publication. I also wish to express my thanks for the facilities given me at the Mount Desert Island Biological Laboratory.

Genus Dahlgrenella

Characteristics: Hydroid stage (1) solitary.

(2) single whorl of oral tentacles.

(3) whorl of basal tentacles ringed with nematocysts.

(4) clusters of medusæ buds just oral to basal tentacles.

(5) asexual reproductive bodies found in perisarc posterior to foot.

[1] Netting is slowly dragged over the bottom. A piece 2 by 4 feet is a convenient size with a one-half inch mesh. The forward edge is fastened along a board 2 feet by 2 inches to which is lashed a 2-foot length of one-inch copper pipe.

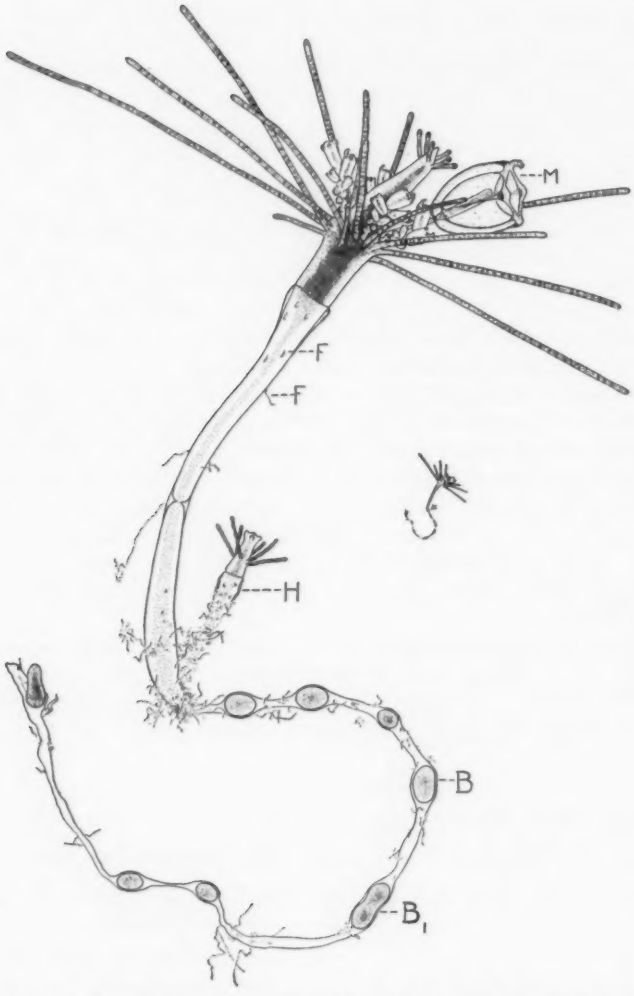

PLATE I. *Dahlgrenella farcta*, mature hydroid with medusæ, *M*, and reproductive bodies, *B*. Reproductive body dividing, B_1. Young hydroid, *H*, probably produced by reproductive body and adhering to hydrorhiza. Frustules, *F*.

Medusa stage (only young medusæ observed)
(1) single short tentacle.
(2) tentacle bulbs with red pigment.
(3) nematocysts scattered over bell.

Size: Hydroid stage—about 5 mm. exclusive of hydrorhiza.
Medusa stage—young individuals about 1 mm.

Found: Mount Desert Island, Maine; on mud bottom between 40 and 60 feet.

Genotype: at the Smithsonian Institute of the National Museum, Washington, D. C.

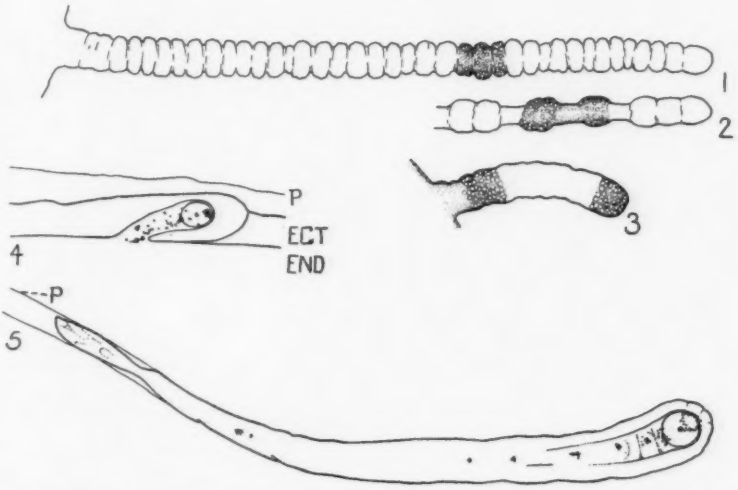

PLATE II. *Dahlgrenella farcta,* basal tentacle, contracted, 1; basal tentacle, expanded, 2; oral tentacle, 3; young frustule, 4; fully developed frustule, 5 (perisarc, *P;* ectoderm, *ECT;* entoderm, *END*).

The hydranth is approximately 5 mm. long exclusive of hydrorhiza which may extend 15 mm. or more. There is a ring of from 6 to 8 capitate oral tentacles around the hypostome as well as a whorl of from 8 to 16 filiform basal tentacles (Plate II). The nematocysts are scattered evenly over the former while they are arranged in rings along the whole length of the latter. The body of the hydranth is also evenly covered with nematocysts. The hydranth extends aborally as a sort of foot secreting the perisarc. Posteriorly the perisarc forms a sticky hydrorhiza enveloping a string of ovoid bodies. The hydranth is pink-

ish or creamy white in color and an opaque white area is present within the ring of oral tentacles.

Frustules somewhat similar to those of *Corymorpha* can be seen on the foot and posterior region of the hydranth (Plates I and II). These develop into tentacle-like processes of adhesive nature much thinner than the oral and basal tentacles and covered with perisarc. The tip may adhere to some surface such as the bottom of a glass container and the cells connecting it with the foot will degenerate leaving only the strand of perisarc as anchorage. The young frustules just below the anterior edge of the perisarc are short protrusions while the more posterior frustules form the long anchorage fibers which may extend 3 mm. or more from the foot.

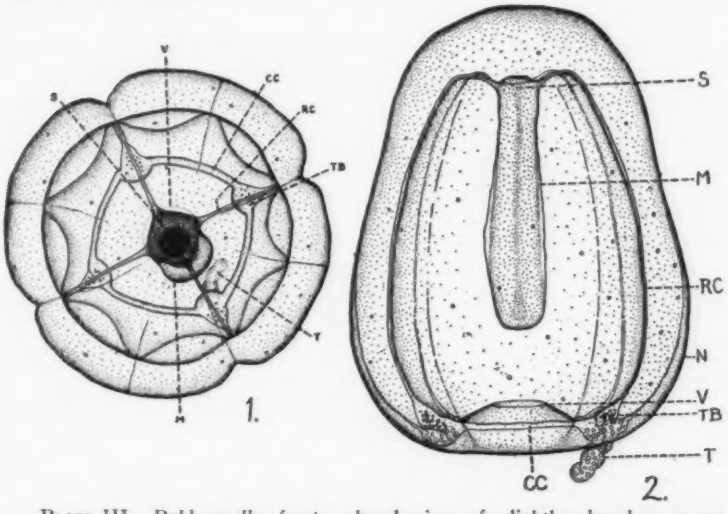

PLATE III. *Dahlgrenella farcta*, aboral view of slightly shrunken young medusa, 1; side view of normal young medusa, 2; circular canal *CC*; radial canal, *RC*; manubrium, *M*; stomach, *S*; single tentacle, *T*; tentacle bulb, *TB*; velum, *V*; nematocyst, *N*.

It is convenient to keep the living specimens for the examination of frustules and reproductive bodies on 75 by 50 mm. glass slides in stacking finger-bowls. The frustules will anchor the hydranth to the slide overnight so that they may be taken from one finger-bowl to another for changing the water and may be placed in Petri dishes for examining under the microscope without greatly disturbing them. By this means the attachment and movements of the more elongate frustules may be studied.

Clusters of medusæ (Plate III) and medusæ buds are located above

the ring of basal tentacles. The young medusæ are about one mm. long when first set free. A single short tentacle densely covered with nematocysts is present. The four tentacle bulbs around the rim of the ovoid bell contain red pigment. In addition to those of the tentacle bulbs and tentacle, nematocysts are sparsely scattered over the entire bell.

The ovoid bodies (Plate IV) enveloped in the hydrorhiza are

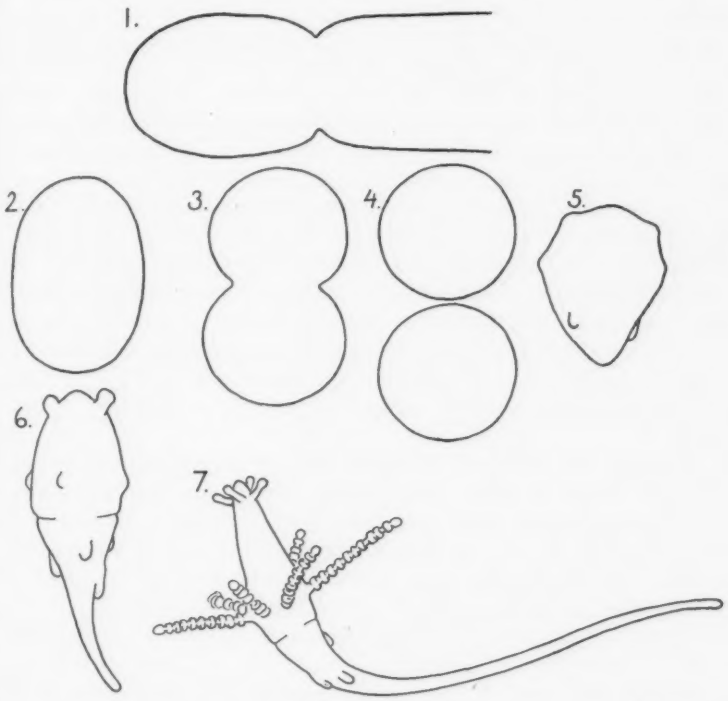

PLATE IV. *Dahlgrenella farcta*, development of asexual reproductive bodies (orientation not shown). Body constricting off from foot, 1; body after constriction (which may be followed by subsequent division), 2; body dividing, 3; body after division (which may be followed by further division), 4; developing hydroid with first appearance of tentacles and frustules, 5; older hydroid with appearance of perisarcal region, 6; older hydroid with well differentiated tentacles, 7. The hydroid usually penetrates the enveloping hydrorhiza between the stages 5 and 6.

formed by fission of the foot and in some cases redivision of the parts constricted off from it. Rudimentary tentacles and frustules appear in

the most posterior bodies first and development proceeds until the young hydroid is formed. As many as twenty bodies, each destined to develop into a new individual, have been observed in a single hydrorhiza.

It may be of some significance in the interpretation of this mode of reproduction that the young individuals appear to develop oriented oppositely from the parent. The hypostomata of the young in most cases point posteriorly in the hydrorhiza. Since two individuals pointed in the same direction may develop from a single body constricting equatorially, it is evident that the orientation is not dependent upon the location of the constriction. The possibility of the presence of an axial gradient should be investigated. However, the bodies become motile early and possibly reverse their positions by their movements. This possibility seems remote since one can observe in the position and structure of the sheath no conditions which might cause the bodies to point uniformly in a direction in which they had not developed.

It is extremely difficult to determine the orientation of the bodies for several reasons. First, the hydrorhiza is often rendered opaque by extraneous matter which has adhered to it. Second, it is impossible to distinguish oral from aboral end until frustules or tentacle buds appear. Third, soon after the appearance of frustules and tentacles the young hydroid penetrates the hydrorhiza thus allowing mechanical influences to disturb its orientation. Therefore this can be determined only under good conditions and only for a short period in the development of the bodies.

However, in a series of 17 specimens kept on glass slides over a period of 4 days, 25 bodies were observed which apparently pointed in the opposite direction from the parent while 2 seemed to point in the same direction. Of 7 other bodies whose orientation was more questionable 6 seemed opposite from the parent and 1 the same. In only 1 case was there evidence that the body had reversed its position. At the end of the 4 days in the 17 specimens there were 133 bodies of which 13 appeared during the last night.

The taxonomy of this form is fairly evident. The arrangement of tentacles in oral and basal whorls with medusa clusters located just above the former clearly indicates its relationship to the genera of the family Corymorphidae. It differs from *Corymorpha* and *Hybocodon* in having but one whorl of oral tentacles. This characteristic it shares with *Ectopleura*. The presence of a single marginal tentacle in the medusa is likewise indicative of its place with the *Corymorphidæ*. In this it is similar to *Hybocodon* and *Corymorpha* but differs from *Ectopleura*. The arrangement of tentacles as well as the distribution of

nematocysts upon them is very similar to *Trichorhiza brunnea* which is generally classified in the family Pennariidæ as well as to *Heterostephanus annulicornis.*

The formation of a new genus for this hydroid seems justified not only because of oral tentacles, size, and form of hydrorhiza but especially because of the unusual method of asexual reproduction. An attempt to identify the medusa stage with any previously described form was also fruitless.

THE RELATION BETWEEN FOOD, THE RATE OF LOCOMOTION AND REPRODUCTION IN THE MARINE AMŒBA, FLABELLULA MIRA[1]

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Introduction

It is known that the rate of locomotion in amœbæ varies with many of the factors in the environment, such as the osmotic pressure (Hopkins, 1928), the hydrogen ion concentration (Pantin, 1923; Hopkins, 1928; Mast and Prosser, 1932; Pitts and Mast, 1933 and 1934), the concentration of salts (Pantin, 1926*a* and *b*; Hopkins, 1929; and Pitts and Mast, 1933 and 1934), the nature of the substratum (Hopkins, 1929), and temperature (Schwitalla, 1924 and 1925; Pantin, 1924; Mast and Prosser, 1932, and Hopkins, 1937). It has been shown, however, that within wide limits the rate of locomotion is not affected by the oxygen tension (Hulpieu, 1930; Pantin, 1930*a* and *b*).

There have been no studies made of the relation of the rate of locomotion to the amount, concentration, or quality of the food which the amœba has had available. Does the ingestion of an abundance of food result in a high rate of locomotion, and conversely, does a scarcity of food result in a low rate of locomotion? Does the nature or quality of the food modify the rate of locomotion? In studies on the rate of locomotion, efforts have been made to maintain the food constant, but a careful consideration of these efforts reveals the fact that they are inadequate if the rate of locomotion is directly dependent upon the food supply. Surprisingly, however, it is observed that regardless of the lack of precise control of the food supply fairly consistent results have been obtained. Could it then be that the rate of locomotion is, within limits, independent of the amount, concentrations and quality of the available food?

The study to be reported here was an attempt to answer some of these questions and to compare this with the effect of food upon growth and reproduction. A marine amœba, *Flabellula mira* Schaeffer was used.

[1] Contribution from the Bermuda Biological Station for Research.

Material and Methods

The amœbæ used in these experiments were from the same clone of *Flabellula mira* Schaeffer, used in recent work on the effect of temperature (Hopkins, 1937). Rice (1935), in a study of the nutrition of this same clone of *Flabellula mira,* found that they could live and reproduce normally and indefinitely on each of several pure strains of bacteria. Consequently I have used, in these experiments, pure strains of bacteria each of which alone would furnish sufficient nutriment for the amœba to live and reproduce normally and indefinitely. Bacteria elaborating substances toxic to the amœbæ were avoided. Six different bacteria were used. They were not identified but isolated, and the fact that they were distinctly different bacteria, established for me by Mr. Jump of the Botany Department of the University of Pennsylvania. They will be designated, Bacterium 1, 2, 4, 5, 7 and 8 respectively.

The experiments consisted of (1) a study of the growth and the rate of locomotion of amœbæ which had been fed bacteria from the pure strains No. 4 in different concentrations, (2) a study of the rate of locomotion of amœbæ fed on two concentrations of each of six bacteria, numbers 1, 2, 4, 5, 7 and 8 respectively, and (3) an experiment on the rate of locomotion in the absence of food particles.

The bacteria were cultured at room temperature in 1 per cent dextrose, 1 per cent Bacto-peptone in distilled water. Each bacterium was inoculated into 100 cc. of this medium, set aside and allowed three days for incubation. At the end of this period the bacteria were centrifuged from the culture medium and washed three times with distilled water. Finally the washed bacteria were diluted with artificial sea water (Butts, 1935) to 100 cc. This stock suspension of bacteria was then diluted as desired with artificial sea water. An equal quantity of each dilution of each bacterium in a Petri dish was inoculated with a definite quantity of culture fluid from a well stirred amœba culture. The same amount of culture fluid was used to inoculate each of the several cultures of a given experiment. One inoculating culture only was used for inoculating all of the cultures of the first set and another for the second set of experiments.

It should be pointed out that by washing the bacteria and finally placing them in artificial sea water, practically all nutrient material for the bacteria was eliminated. Thus growth and multiplication of the bacteria during the experiment was maintained at a minimum.

Method of Measuring the Growth of the Amœbæ in the Cultures

Since the number of amœbæ with which the different experimental cultures were inoculated was approximately the same for each culture,

the concentration of amœbæ in each culture after a given growth period would give a value which would be an index of the relative growth in the culture, i.e., in comparison with the other cultures of the same series. The concentration of amœbæ was obtained by counting the number of amœbæ occurring on a square millimeter of the surface film, plus the number occurring on a square millimeter of the bottom of the dish. In an undisturbed culture all of these amœbæ occur either on the bottom of the dish or on the surface film. In order to obtain a representative value for each culture the number of amœbæ in each of ten surface-films plus bottom-millimeter squares were counted and averaged.

Rate of Locomotion

The rate of locomotion was obtained as described in a recent paper (Hopkins, 1937) except that in those experiments I was able to equalize the error due to the presence of bacteria in the locomotion medium by inoculating equal quantities of culture fluid containing amœbæ and bacteria from the same culture into equal quantities of the locomotion medium. But since the amœbæ in the present experiments must come from cultures containing different concentrations of bacteria, it was not possible thus to equalize this error.

By recording the rate of locomotion only when this amœba has a large clear and smooth hyaline edge, most of the error due to the presence of bacteria or other food particles is eliminated. In feeding, this amœba forms food cups mostly in the hyaline edge. When a food organism is contacted by the hyaline edge, a cup is formed at the point of contact. The protoplasm of the hyaline edge concentrates in the region of the cup and diminishes considerably the area of the hyaline edge. Then the cup with the captured food is drawn into the body of the cell. This process can be detected under low power mainly by the decrease in area of the hyaline edge. Oftentimes, however, the feeding process passes unobserved, resulting in a negative error in the observed rate of locomotion.

By washing the amœbæ several times the bacteria may be eliminated. But this procedure is very tedious due to the small size of the amœbæ, and required for these experiments more time than was available, since it was necessary to measure the rate of locomotion of the amœbæ in all the cultures of a series in as short a time as possible. In view of these considerations, the expedient used to equalize the error was to dilute the drop of culture fluid containing the amœbæ with bacteria-free artificial sea water according to the concentration of bacteria known to be in the

culture, thus approximately equalizing the concentration of bacteria in the locomotion medium at the time when the rate was observed.

Results

The Relation between the Concentration of Food, Growth, and the Rate of Locomotion in Flabellula mira

A stock suspension of Bacterium 4 was diluted 2, 4, 8 and 10 times with artificial sea water and two portions (20 cc. each in a Petri dish) of each dilution inoculated with equal numbers of amœbæ. In addition two cultures were set up by merely adding the same quantity of inoculating fluid and amœbæ to 20 cc. of artificial sea water. All of the cultures were allowed to develop at room temperature (18° to 23° C.).

Table I

Showing the relation between the rate of locomotion and the concentration of Bacterium 4 available as food. X is concentration of bacteria in the stock suspension, X/2, X/4, X/8 and X/10 various dilutions. Concentration y indicates the concentrations of bacteria of cultures to which only the bacteria with the inoculating amœbæ were added. Rate in micra/minutes in artificial sea water. Temperature 21.6° + 0.2° C.

Concentration of Bacterium 4	*X*/2	*X*/4	*X*/8	*X*/10	*y*
Third day		27.0	27.5	28.0	29.5
Fifth day	26.0	30.0	27.0	29.0	29.0
Eighth day	30.0	31.0	33.0	30.0	33.0
Average	28.0	29.3	29.2	29.0	30.5
Average for all experiments	29.2				

On the third, fifth and eighth days samples of amœbæ were removed from each culture and diluted with artificial sea water, as described above, and the rate of locomotion determined for five minutes for each of the five amœbæ at 21.6 ± 0.2° C. The results for each culture were averaged and this average was averaged with the other culture of the same dilution. The results are presented in Table I.

On the seventh day the concentration of amœbæ in each culture was observed and the results for each culture averaged with results from the other culture of the same dilution. The results are presented in Table II.

From the results presented in Table I it is quite clearly shown that within the limits here studied the concentration of bacteria which has been available to the amœbæ as food has no influence on the rate of locomotion.

From the results presented in Table II it is shown that the growth of these amœbæ increases as the concentration of the bacteria increases. The highest concentration of bacteria used here gave the greatest, and the lowest concentration the least amount of growth.

The Relation between the Kind and the Concentration of Bacteria Available as Food and the Rate of Locomotion

In these experiments the amœbæ were fed on two concentrations of each of the six bacteria 1, 2, 4, 5, 7 and 8. The amœbæ were inoculated into 20 cc. of the stock suspension of each bacterium prepared as described above and into 10 cc. of the stock suspension diluted to 20 cc. with artificial sea water. The rate of locomotion, in artificial sea water at 21.6 ± 0.2° C., of amœbæ fed for five days on each concentration of

TABLE II

Showing the relative growth of amœbæ fed on different concentrations of bacteria for seven days. The concentrations of the bacteria are the same as in Table I. Temperature the same for all cultures. Amœbæ per sq.mm. of bottom + sq.mm. of surface film.

Concentrations of bacteria	*X*/2	*X*/4	*X*/8	*X*/10	*y*
Culture I	25.0	9.2	9.0	10.0	8.2
Culture II	18.0	28.8	12.6	7.9	5.6
Average	21.5	19.0	10.8	8.9	6.9

bacteria, was obtained by averaging the results of five minutes locomotion for each of five amœbæ. The results are presented in Table III.

From the results of Table III it is observed that the rate of locomotion of these amœbæ has not been influenced by the variations in kind and concentration of the bacteria on which they have been fed.

The Rate of Locomotion of Flabellula mira in Artificial Sea Water at 21.6 ± 0.2° C. and its Constancy When All Food Particles are Absent from the Medium

By a food particle is meant here any particle which the amœba will ingest. Since, as has been shown, the rate of locomotion of *Flabellula mira* is within wide limits independent of the amount and kind of food it has had available, it seemed worth while to determine accurately the rate under definite and reproduceable conditions. Also the question arose as to whether the rate of locomotion in the absence of external stimulation is variable or constant. Schwitalla (1924) has maintained

that locomotion in *Amœba proteus* is never constant but varies in a rhythmical fashion. Hahnert (1932) and Pitts (1933), however, have shown that Schwitalla's rhythms were due to the fact that in measuring the rate he did not take into consideration the changes in form of the amœbæ while observing the rate. They maintain that in *Amœba proteus* when the form is monopodal the rate of locomotion is constant.

In the experiment now to be described, the object was to remove from the environment of the amœbæ, as much as possible, all chances of stimulation from the environment. The main and most frequent stimulation from the exterior to which they react is to particles which they take in as food. They engulf not only bacteria but in addition yeast, carbon particles, dust, etc. In order to eliminate interference to locomotion due to such particles the following procedure was followed.

TABLE III

Showing the relation between the kind and concentration of bacteria and the rate of locomotion. X is concentration of bacteria in the stock suspension, and X/2 the dilution. Rate in micra/minutes in artificial sea water. Temperature 21.6 ± 0.2° C.

Bacterium No.	1	2	4	5	7	8
X	28.0	27.0	30.0	28.0	32.0	30.0
X/2	32.0	28.0	30.0	26.0	29.0	28.0
Average	30.0	27.5	30.0	27.0	30.5	29.0

Average for all experiments 29.0

The Pyrex glass dishes, or locomotion dishes, were washed in sulfuric acid-potassium bichromate cleaning solution, rinsed several times in distilled water, dried, polished with a clean cloth and placed on a sheet of platinum and heated to a red heat for about ten minutes. In this way the surface of the dish was freed of all organic particles. The artificial sea water was filtered through three layers of No. 50 Whatman filter paper, after the filter paper had been washed thoroughly by running through it several hundred cubic centimeters of artificial sea water. The amœbæ were washed free of bacteria by removing them singly from the culture with a sterile capillary pipette and passing them through five changes of the filtered artificial sea water. They were finally placed in filtered artificial sea water in the cleansed Pyrex glass dish. The dish was then covered with a thoroughly cleansed cover-slip to keep out dust from the air. The dish containing the amœbæ was placed in the observation chamber of the constant temperature apparatus

(Hopkins, 1937) at $21.6 \pm 0.2°$ C. Cultural temperature for the amœbæ used was from 18° to 22° C. Thus no adaptation to temperature (Hopkins, 1937) was necessary before locomotion could become constant. One hour, however, was allowed for the amœbæ to recover from the effects of handling and the small change in light intensity associated with the artificial light used for the observations. The environment thus established was remarkably free of external stimulating agents. The medium, however, still contained a few bacteria and small particles which could be seen under low-power of the microscope. These were very infrequent such that they could be detected before the amœbæ under observation contacted them. The performance of each of three amœbæ was observed. The observation on each amœba was continued until it contacted and engulfed a visible particle which resulted in a temporary cessation of locomotion. During the periods of

TABLE IV

Showing the performance of three amœbæ in the absence of food particles. Temperature 21.6° C.

Amœba	Distance in mm. traveled by amœbæ in each of a number of consecutive minutes × 118	Rate micra/min.
A	4, 4, 3, 4, 3, 4, 4, 4, 4, 4, 4, 3, 4, 4, 4, 5, 5, 4, 4, 4, 4, 4, 4, 4, 5, 3, 4, 4, 4, 4, 4, 4, 4, 5, 4, 4, 4, 4, 4, 4, 4, 4, 3, 4, 5, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4.	33.9
B	3, 4, 4, 5, 4, 4, 4, 4, 4, 3, 3, 3, 3, 3, 4, 4, 4, 5.	31.9
C	4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4.	33.9
Average		33.23

observation these amœbæ contacted a few particles which they did not engulf, and no interruption in the locomotion occurred. Amœba A traveled 55 minutes before it engulfed a particle and ceased temporarily to move; B 18 minutes, and C 25 minutes. The minute to minute performance of each of these three amœbæ is given in Table IV. In the table the distance traveled each minute was measured to the nearest magnified millimeter.

The results show that no rhythms occur in the rate of locomotion of this amœba and that as long as it received no stimuli from the environment, its rate of locomotion is constant.

When we average the rates obtained in the preceding experiments, a value of 29.1 micra/min. is obtained for the rate of locomotion of these amœbæ in artificial sea water at $21.6 \pm 0.2°$ C. and in the presence of a relatively few food particles. This is 4.13 micra/min. low when compared with the rate obtained in the last experiment, a negative error of

aproximately 13 per cent. This error is undoubtedly due to the presence of food particles in the environment, and appears to be unavoidable if food particles are present in any abundance. The interference to locomotion due to the presence of food probably occurs only after the amœba has come in contact with the food since Rice (1935) has shown that this amœba cannot sense its food at any distance. After ingestion of food it is possible that the amœba does not immediately resume its normal rate but accelerates slowly.

Discussion

These results appear to be very definite and far-reaching in their implications. It must be pointed out, however, that they cannot be taken to mean that food has no influence upon the rate of locomotion. Food, of course, must be available to furnish the energy necessary for locomotion. The concentrations of food studied in these experiments do not include extremely high or low concentrations. It is very probable that in these amœbæ when the food becomes scarce and the energy necessary for locomotion is no longer available, the rate of locomotion is lowered. It is also possible that when the food organisms become too plentiful the excretion products become sufficiently concentrated to affect the rate of locomotion. It was felt, when these experiments were planned, that the higher concentrations used were sufficient to produce an effect on the rate of locomotion. That this was not the case has been seen. Repeated efforts have been made to actually starve these amœbæ, but the technique involved is very difficult and a satisfactory method has not yet been devised. It may be that starvation to such an extent that the energy necessary for locomotion is not available, results in a rapid decrease in the rate of locomotion to zero, followed immediately by encystment, or that encystment may take place before the available energy has fallen to such a low level.

It is quite evident, however, that when an excess of food is ingested by this amœba it is not utilized by an increase in the rate of locomotion but is utilized by an increase in the rate of growth and reproduction. This procedure, of course, has a distinct survival value. On the other hand, the fact that when food becomes relatively scarce the rate of locomotion is not decreased has a survival value in that the amœbæ are better able to reach regions where food is more abundant. Failing of this the most expedient reaction, viewing the matter in the light of possible survival, would be to encyst and remain thus until food in the environment is again plentiful. It has been shown (Beers, 1926) that a deficiency of food results in encystment in *Didinium*.

With reference to the possibility that these findings for the relation of the rate of locomotion to food in *Flabellula mira* hold for the other amœbæ, it might be said here that experiments now in progress indicate that they hold for fan-shaped amœbæ but not for limacine amœbæ.

Conclusions

1. The rate of locomotion of *Flabellula mira,* within limits, is independent of the amount of food it has had available.

2. Growth and reproduction of *Flabellula mira* are dependent on the concentration of the food available.

3. The rate of locomotion of *Flabellula mira* is independent of the kind of bacteria available as food, provided the bacteria are adequate for normal growth and reproduction.

4. In the absence of external stimuli and in a normal environment to which it is completely adapted, the rate of locomotion in *Flabellula mira* is constant, as far as can be observed under a magnification of 118 diameters, and is very close to 33 micra per minute at 21.6° C.

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THE CHROMATOPHOROTROPIC HORMONE OF THE CRUSTACEA: STANDARDIZATION, PROPERTIES AND PHYSIOLOGY OF THE EYE-STALK GLANDS [1]

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STANDARDIZATION

The need for a method of determining quantitatively the amount of hormone in a given extract of a gland is obvious in chemical or quantitative physiological work dealing with endocrine systems. Although the existence of such a system for the pigmentary effectors of crustaceans has been known for almost ten years, no reliable method for the standardization of the eye-stalk hormone has been devised. Navez and Kropp (1934), and Kropp and Crozier (1934) suggested that the eye-stalk hormone may be standardized by the *Avena* coleoptile method since they found that the eye-stalk extract accelerates the growth rate of *Avena* coleoptiles. These authors employed water extracts of the eye-stalks of *Palæmonetes*. Since many other substances in addition to the chromatophore hormone are present in a water extract of the eye-stalks, it is not certain that the growth effects on plants are due actually to the chromatophore hormone. On the basis of biochemical evidence, Carlson (1936) suggested that the growth reactions of plants to the eye-stalk extract was due to the presence of some substance other than the chromatophore hormone. If this is true, it would then appear that Kropp and Crozier have not been measuring the chromatophore hormone but possibly some other substance present in a water extract of the eye-stalks.

Consequently, it was felt that the most reliable way of assaying the chromatophore hormone would be to measure the response of the tissue (the chromatophores) which this hormone normally affects.[2] A convenient laboratory animal on which a method of assay may be devised is the fiddler crab, *Uca*. In the vicinity of Woods Hole, two species, *U. pugilator* and *U. pugnax*, are abundantly present. Both these spe-

[1] Aided in part by a grant from the Rockefeller Foundation, administered by F. L. Hisaw.

[2] Cf. Burn, J. H., 1930. *Phys. Rev.*, **10**: 146.

cies contain black, red, white and yellow integumentary chromatophores. Only occasionally, however, are red chromatophores observed in *U. pugnax*. The extreme color change of *U. pugnax* ranges from jet black to pale yellow (Figs. 3, 4) and of *U. pugilator* from dark brown to cream white (Figs. 5, 6). The melanophores are the chief agents of this change although the movements of the other pigments are instrumental in producing the end result. Megašur (1912) demonstrated that extirpation of the eye-stalks of *Uca* resulted in the production of the pale phase of its color change due to melanophore contraction. Carlson (1935, 1936) confirmed this observation and interpreted it as an effect specifically due to the loss of the endocrine glands contained in the eye-stalks. An extract of the extirpated eye-stalks when injected into a pale (blinded) animal produced the dark phase resulting from melanophore expansion. Carlson showed further that if 0.1 cc. of a solution containing the extract of 1 eye-stalk of *Uca* in 1 cc. of water was injected into a blinded [3] crab, the melanophores became expanded within 2 hours, remained expanded for about 4 hours, and finally contracted so that the animal resumed the pallor previous to injection. This is specifically a hormonal reaction. There appears to be no other way whereby the melanophores of a blinded *Uca* may be induced to expand.

Preliminary Experiments

The statements of Megašur and Carlson concerning the effects of blinding and of injection of eye-stalk extracts were first confirmed (Figs. 3, 4, 5, 6). The typical response of a blinded animal following the injection of an extract of its eye-stalks in a dose equivalent to 1/20 of a single stalk may be conveniently divided into four phases:

(*a*) The first perceptible response.

This response is evidenced by the beginning of melanophore expansion and occurs from 15–20 minutes after the injection has been made.

(*b*) The attainment of the maximal effect.

After the first perceptible response has occurred, the melanophores become maximally expanded within one hour.

(*c*) Duration of melanophore expansion.

Following the attainment of the maximal effect, the melanophores remain fully expanded for about 3¼ hours.

(*d*) The period during which the melanophores contract again.

Four and one-half hours following the time of injection, the

[3] The term *blinded* will be used throughout this paper to designate the condition of specimens whose eye-stalks have been completely removed.

melanophores begin to contract, and within the following half-hour, the animal becomes again pale, so that the melanophores are in the same condition as that previous to the injection.

Preliminary experiments were therefore designed to determine the relation between each of the four phases of the response and concentration of hormone. A sea water extract was prepared from 280 eye-stalks of *Uca pugnax* and a series of the ten following dilutions was made: 8, 4, 2, 1, 0.5, 0.25, 0.12, 0.06, 0.03, 0.015 E.S.[4] per cc. of solution. Several hundred specimens of *Uca pugnax,* blinded two days previously, were employed as test animals. All animals were of equal size and weight (3.5 grams). The injection volume in all experiments was 0.1 cc. Fifty animals in groups of 5 constituted the experimental material for each test. The tests were carried out by injecting each group of 5 animals with but one of the 10 dilutions so that each animal in the first group received a dose of 0.8 E.S. in the second group 0.4 E.S., in the third 0.2 E.S., etc. It was found that the time at which the first perceptible response occurred was independent of the concentration of hormone. In all ten groups the first signs of melanophore expansion occurred 15–20 minutes following the time of injection, although at the two lowest concentrations (0.0015 E.S. and 0.003 E.S.) this time was slightly increased. The attainment of the maximal effect was likewise found to be independent of concentration when concentrations higher than 0.003 E.S. were used. Concentrations below this value did not produce the maximal response. The duration of melanophore expansion, however, was found to be proportional exponentially to the concentration of hormone employed (doses above 0.003 E.S.). The time during which the melanophores became contracted again was, like the first two phases of the response, independent of the concentration.

The significant point of these experiments is the demonstration that the duration of melanophore expansion varies as a function of concentration. The times for the completion of the other three phases of the response are for all practical purposes constants, independent of concentration provided the doses employed are greater than 0.003 E.S. In order to measure the response of the animals to a given concentration, it is therefore necessary to determine only the time of injection and the time at which the animals become again pale. The difference in these two readings is obviously the duration of the response plus 1.75 hours,

[4] The letters E.S. will be used throughout this paper as an abbreviation for the extract equivalent to a given number of eye-stalks. For example, 1 E.S. indicates that the extract is equivalent to that obtainable from one eye-stalk; 0.5 E.S. denotes an extract equivalent to half that obtained from one eye-stalk, etc.

which may be used as a constant equal to the sum of the times of the other three phases of the response.

Variables in the Method of Assay

There are several important variables which must be controlled completely if such a method of assay is to be used.

(1) *Physiological Uniformity of the Responsive Tissue.*—The black chromatophores were used as the sole criterion in obtaining readings since they are the chief instruments in determining the appearance of the animal with respect to paleness or darkness. In blinded specimens of *Uca* the pigment in all melanophores of the appendages and body is uniformly and extremely concentrated, and remains so indefinitely regardless of environmental conditions which would provoke expansion in a normal animal. Thus it appears certain that the responsive tissue in all test animals is in a physiologically uniform state.

TABLE I

Size of eye-stalk in relation to hormone concentration

Size of Eye-stalk	Injected Dose	No. Tested	Average Weight of Test Animals	Average Response
			grams	*hours*
Small (animal weight, 1.7 grams)	0.025 E.S./cc.	8	3.5	3.75
Medium (animal weight, 2.8 grams)	0.025 E.S./cc.	8	3.6	4.09
Large (animal weight, 5.1 grams)	0.025 E.S./cc.	8	3.5	4.88

(2) *Concentration of the Injected Hormone.*—This variable is easily controlled since the method of preparing the extract unless otherwise stated was maintained constant. The eye-stalks were cut off and ground in a small mortar in the amount of sea water necessary for any particular concentration. This extract was brought to a boil so that a coagulum (presumably the tissue proteins) formed. The solution was filtered, and the filtrate made up by the addition of unboiled sea water to the desired concentration. About 0.6 cc. of the sea water was usually lost during the process of boiling and filtration. This resulted in a slightly hypertonic extract, but this factor is immaterial for the purpose of the experiment (cf. Carlson, 1936). The solution was allowed to cool to room temperature, usually about one-half hour elapsing, and the injection made. While the concentration of the hormone in terms of numbers of eye-stalks can be controlled quite accurately, the size of the eye-stalks used in making an extract of a certain concentration could not be controlled so easily. Larger eye-stalks may contain large glands and hence more hormone, as shown by Table I. To control this factor,

the extracts were prepared from eye-stalks of animals of a definite and uniform size.

(3) *Size of the Test Animal.*—This variable proved to be a very disconcerting factor at the beginning of the work. It was reasonable to suppose that large animals would show a smaller response to a given dosage than small animals because they would contain a greater amount of the responsive tissue, and because the hormone would be subjected to greater dilution. That this is plausible is shown by Table II.

Consequently, in order to avoid the use of a curve for the calibration of response as a function of the size of the test animals, assay experiments were always carried out on animals of uniform weight.

(4) *Individual Variation.*—This variable is probably the most significant of those already mentioned because it is not easily controlled. Since it is almost impossible to carry out all experiments on the same animal, the best method would be to use sufficient numbers of animals

TABLE II

Size of test animal in relation to response

Hormone Concentration	No. of Animals Tested	Average Weight	Average Response
		grams	*hours*
1.0 E.S./cc.	10	4.36	4.13
1.0 E.S./cc.	6	3.86	5.56
1.0 E.S./cc.	10	1.55	7.83

and to treat the data statistically. In performing an assay, from 15 to 20 animals of the same size and weight were injected with the same volume and concentration of hormone and the average response taken. The standard deviation of the arithmetic mean was calculated and determinations made to see if the differences in the response at different concentrations were really significant.

(5) *Volume of Injected Dosage: Place of Injection.*—Several experiments made to determine the most suitable volume of hormone to be injected led me to adopt finally 0.05 cc. as the standard injection volume. Carlson used 0.1 cc. but while this is satisfactory for *U. pugnax* and very large specimens of *U. pugilator,* it seemed to be too great a dose for the size of crabs employed in most of my experiments. The most convenient region for the injection of the hormone into the body spaces was found after many trials on different regions of the body to be through the soft tissue forming the joint between the coxipodite and the protopidite of the walking legs. This method allows for speed and

accuracy of injection for, using a 27-gauge hypodermic needle, one can inject 20 crabs within 2 minutes.

Method Finally Adopted

Upon the basis of these preliminary experiments, the standardization of the eye-stalk hormone was completed using blinded *Uca pugilator* [5] as a test animal. One hundred and fifty such specimens, whose eye-stalks were extirpated two days previously, were arranged in ten groups of 15 animals each. All animals were of uniform size and weight ($2.46 \pm .01$ gram). A stock solution of hormone was made by extracting the extirpated eye-stalks as previously described. A series of 10 concentrations ranging from 5 E.S./cc. to 0.03 E.S./cc. was prepared. Five-hundredths of a cc. of these concentrations was used as the injection volume in all cases. Each of the 15 animals in a particular group received 0.05 cc. of a particular concentration, so that each of the ten groups of animals was injected with but one of the ten various known concentrations. The difference between the time of injection and time at which the animals became pale again was noted. The results of this experiment are shown by Fig. 1. The experiment was repeated five times, and although the points obtained did not fit the curve as well as those of Fig. 1 do, it was generally true that the magnitude of the response was exponentially proportional to concentration.

It can be seen from Fig. 1 that the relation between the complete response of the melanophores and the concentration of hormone is least sensitive over the range of concentrations above 1.0 E.S./cc. and most sensitive over the range from 0.06 E.S./cc. to 1.0 E.S./cc. This sensitive range is re-plotted in Fig. 2, which shows that there is a linear relationship between the duration of melanophore expansion and concentration of hormone.

Figure 2 was therefore used to establish the *Uca* unit. Two possibilities are open. One can choose an arbitrary response near the midpoint of this curve and designate this value as one *Uca* unit. Assays would therefore be made by repeated dilutions of an unknown until the resulting response equals that chosen to represent one *Uca* unit. A second possible method would be to inject 0.05 cc. of an unknown concentration, obtain the average response and read off from Fig. 1 the concentration in terms of *Uca* E.S./cc. which gives the same response. The latter procedure was adopted because both amount to the same thing provided the unknown concentration falls within the sensitive

[5] *Uca pugilator* was substituted for *U. pugnax* as the standard test animal because it is much more uniform in weight and size and because the rate of mortality and the frequency of autotomy are much lower than in *U. pugnax*.

range.[6] The response (4.9 hours) produced by 0.05 cc. of an extract of 1 E.S. of *U. pugilator* (2.46 grams) per cc. of solution was designated as one *Uca* unit.

Hormone Content in the Eye-Stalks of Various Crustaceans

In estimating the hormone content in the eye-stalks of various crustaceans, the following method was used: a number of eye-stalks

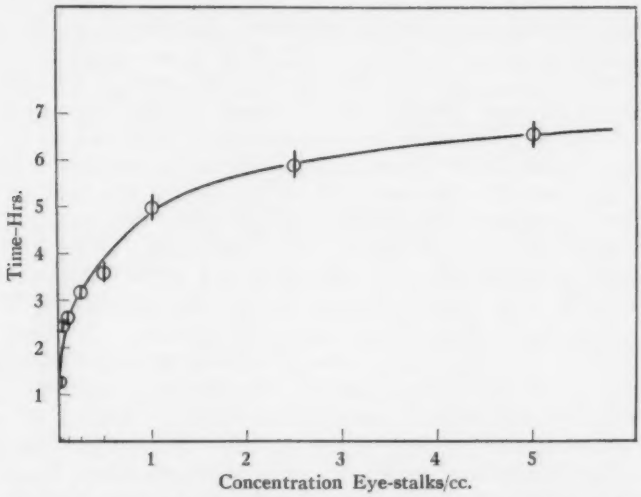

Fig. 1. Curve showing the relationship between the concentration of the hormone and the complete response of *Uca pugilator*. The points are averages of 15–20 animals, over 150 animals being used in this particular experiment. The solid bars represent values equal to twice the standard deviation of the arithmetic mean.

were cut off, extracted with sea-water, boiled, filtered, and in all cases the solution was made up so that 1 cc. contained the extract equivalent

[6] This range is sensitive enough to detect differences in response produced by a concentration of X, and one of 2X. The significant differences between some of the points along this range are much greater than that required by the standard equation $\frac{S.E._{.1} - S.E._{.2}}{\sqrt{\sigma^2_{.1} + \sigma^2_{.2}}} = \text{or} > 3$. The sensitivity of the curve would be increased by obtaining more points along this range, and by using extremely large numbers of animals to cut down the size of the standard error.

to 1 eye-stalk.[7] Five-hundredths of a cc. of the solution was injected into each of 15 animals and the average of the responses taken. The concentration in terms of *U. pugilator* E.S./cc. which produces the same average response was read off from Fig. 1. The hormone content of one eye-stalk of various crustaceans as compared with one eye-stalk of *U. pugilator* (1 unit) is given in Table III. The extracts were always made during the day and from animals which had been maintained under illumination (daylight).

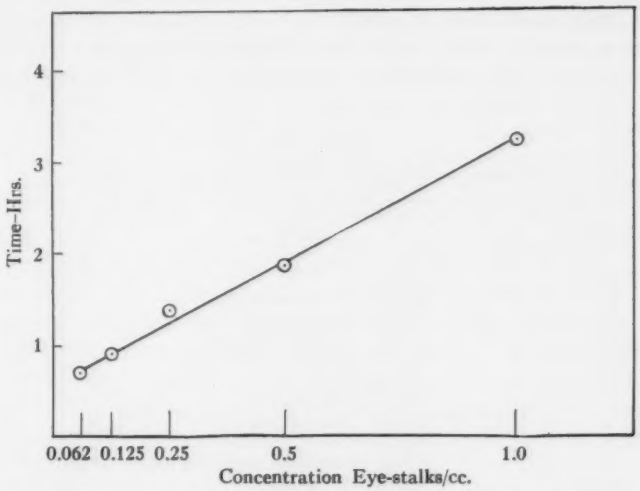

FIG. 2. Curve showing relationship between the duration of melanophore expansion and concentration of hormone (values replotted from Fig. 1). Theoretically, the curve should go through the 0 point.

EXTRACTION AND PURIFICATION

Although ten years have elapsed since the eye-stalk hormone was discovered, very few chemical properties of the hormone are known. Perkins showed that the hormone was soluble in water, and that it was resistant to boiling. Carlson (1936) and Abramowitz (1936*a*, *c*) demonstrated that it was soluble in alcohol but insoluble in ether. Carlson also added that the hormone was not destroyed when treated for short periods of time with acid or alkali.

[7] Unfortunately this proved to be a mistake because a few readings obtained in assaying the potency of the eye-stalk extracts of certain crustaceans were not within the sensitive range, and therefore cannot be considered as accurate readings.

Since a method for standardizing the hormone has been devised, an attempt was made to purify the substance. One thousand eye-stalks of *Uca pugilator* were dried and pulverized. The total activity of the dry powder was 1000 *Uca* units, or 0.6 *Uca* units per mg. of dry powder. This material was extracted several times with small volumes of light petroleum ether in order to remove a red carotenoid pigment which was found to contaminate the subsequent fractions. The ether solution was washed with small amounts of distilled water. The water layer was then added to the residue insoluble in ether and the ether layer being only slightly active was discarded. The residue which was insoluble in ether was then extracted three times with distilled water. The water solution was boiled and filtered, and the filtrate dried in a current of

TABLE III

Hormone content of the eye-stalks of various crustaceans

Average Weight of Animals	Species	*Uca pugilator* Units
grams		
2.46	1 E.S. *Uca pugilator*	1.0
3.3	1 E.S. *Uca pugnax*	1.0
11.0	1 E.S. *Pagurus pollicaris*	1.25
309.0	1 E.S. *Homarus americanus*	1.20
2.0	1 Head *Hippa talpoida*	0.25
0.38	1 E.S. *Crago borealis*	0.25
219.0	1 E.S. *Carcinus mænas*	1.25
80.0	1 E.S. *Cancer irroratus*	5.0
0.4	1 E.S. *Palæmonetes vulgaris*	0.36
15.0	1 E.S. *Uca minax*	1.5
127.0	1 E.S. *Libinia dubia*	4.0

warm air. The dried material was washed with small amounts of chloroform to remove traces of the red pigment, and the chloroform washings being only slightly active were discarded. The dried filtrate was then extracted with 95 per cent ethanol, and the alcohol solution was centrifuged. The alcohol-soluble fraction was decanted and dried. Both the alcohol-soluble and alcohol-insoluble fractions were active, the latter being much less so than the former, and hence was discarded. The material soluble in 95 per cent ethanol was then dissolved in hot absolute alcohol and precipitated by the addition of ether. The activity of the material soluble in absolute alcohol was approximately two *Uca* units per mg. Further attempts to precipitate the material proved unsatisfactory because of the exceedingly small yield. The loss in total

activity was about 60 per cent. The material which is soluble in absolute alcohol is devoid of pigment and is apparently protein-free.[8]

Properties of the Hormone

The eye-stalk hormone is readily soluble in water, but not completely soluble in ethanol or methanol. It is only slightly soluble in acetone, and insoluble in organic solvents such as benzin, chloroform, or ether. The hormone is thermostable, but is destroyed by oxidation. It does not decompose when boiled with HCl or NaOH in a 1 per cent solution for short periods of time. If the hormone is boiled for 2 hours with NaOH, the activity is completely destroyed. The hormone adsorbs easily to various substances present in crude extracts of the eye-stalks. If the eye-stalks are extracted with benzin, and the benzin-soluble material (chiefly pigment) and the benzin-insoluble material tested in equivalent doses, only the benzin-insoluble is found to be active. However, if the benzin-soluble material is concentrated and then tested, some activity will be found. The same phenomenon was observed when working up sea-water extracts of the eye-stalks of various crustaceans. These extracts were dried, and the dried material extracted with 95 per cent ethanol. Two volumes of acetone were then added to the alcohol solution to precipitate the salts, which settle out in crystalline form. The salt crystals were also found to be slightly active. The observations indicate that the hormone adsorbs very readily. The hormone may be kept in a water solution in the refrigerator for some time without appreciable loss of activity. However, it is destroyed slowly when kept in a water solution at room temperature.

Physiology of the Eye-Stalk Glands

One of the basic problems in endocrine research is to determine the factors which affect the production of a hormone and the mechanism which regulates its release into the circulation. The standardization of the eye-stalk hormone has made it possible to investigate the physiology of the eye-stalk glands, for the amount of hormone in the stalks of animals maintained under special conditions can now be determined. The presence or absence of the hormone in the circulation can be determined quite easily by observing the states of the chief chromatophores with the aid of a microscope. This can be illustrated by the pigmentary reactions of *Uca* and *Palæmonetes,* the two animals chosen for this investigation. Perkins (1928) and Brown (1933) have shown that the contraction of

[8] The alcohol-soluble material gave negative results when tested with Millon's reagent and with the Xanthaproteic test.

the red and yellow chromatophores of *Palæmonetes* is due to the presence of the hormone in the blood stream, and that the expansion of these chromatophores results from the absence of the hormone. The situation is just reversed in *Uca* for in the brachyurans the eye-stalk hormone expands the melanophores and the erythrophores when it is circulating through the animal, while its absence results in the contraction of these chromatophores.

Palæmonetes

Forty specimens of *Palæmonetes vulgaris* of uniform size and weight were separated into four equal groups. One group was placed in a black vessel, another in a white, a third group in a yellow and a fourth in a blue. The vessels were then placed under the illumination of two 75-watt electric bulbs. The animals were supplied with a continuous current of fresh sea water, and left undisturbed for 8 hours. A fifth group of 8 animals were placed in total darkness for a day. At the end of 8 hours, the eye-stalks of each group under illumination were extracted with sea water and each of the 4 extracts assayed to determine the amount of hormone present. The eye-stalks of the animals placed in total darkness were extracted in darkness on the following day. The results of this investigation are listed in Table IV.

TABLE IV

Hormone content in the eye-stalks of Palæmonetes under various environmental conditions

Number of Eye-stalks Tested	Condition	Average Weight of Animals	*Uca* Units/E.S. of Palæmonetes
		grams	
20	Black-adapted	0.25	0.46
20	White-adapted	0.29	0.50
20	Yellow-adapted	0.30	0.49
20	Blue-adapted	0.25	0.47
16	Darkness (day)	0.28	0.25

This experiment was repeated three times and the same result was obtained in each case. The amount of hormone present in the eye-stalks of animals kept under illumination was twice that obtained from animals maintained in darkness. This is confirmatory of Kropp and Crozier's finding that stalk extracts of animals kept in darkness did not depress the growth rate of *Lupinus* as much as extracts made from animals exposed to light. It is also in agreement with the results of Klein-

holz who found that extracts from animals kept in the darkroom produced weaker responses in the retinal pigments than extracts of animals under illumination. Equally significant is the finding that the amount of hormone in the eye-stalks of animals showing a continuous release of the hormone (white-adapted group) was the same as that of animals showing no, or a subminimal release of the hormone into the circulation (black-adapted group).

This situation becomes understandable when the functional cycle of an endocrine gland is considered. The normal physiology of an endocrine gland consists chiefly in synthesizing and storing a hormone, and finally releasing it into the circulation. These three processes may conceivably be controlled separately, or may be controlled uniformly or in part by the same mechanism. In *Palæmonetes,* as in some other crustaceans, the endocrine glands (Blutdrüse of Hanstrom, 1934) of the eye-stalk are innervated from the cerebral ganglia. The functional innervation of a gland would therefore afford a simple mechanism for the release of the hormone into the circulation.

The results listed in Table IV can be readily explained upon the basis of relative rates of hormone synthesis and hormone release. In darkness, there is no release of the hormone into the circulation as indicated by the inactive position of the distal pigment cells of the retina, and by the expansion of the red integumentary chromatophores (Brown, 1935*b*).[9] The rate of synthesis of the hormone must also be decidedly reduced as evidenced by the assays of the eye-stalks. In the presence of light, however, hormone synthesis is greatly increased, and this effect is produced regardless of the background over which the animals are kept. Hormone synthesis, therefore, is due to incident light and is not primarily dependent on reflected light. This accelerating effect of incident light may therefore be termed the primary effect.

The release of the hormone, however, is definitely brought about by light reflected from backgrounds such as yellow or white. This is shown by the contraction of the red and yellow chromatophores of the integument and by the inward migration of the distal cells of the retina under such conditions. The release of the hormone into the circulation is maximal. However, in the black-adapted animals there is no release or only a sub-minimal release of the hormone into the circulation as

[9] The condition of the red pigment in *Palæmonetes* maintained in darkness does not seem to be definitely settled. Perkins (1928) stated that the red pigment was concentrated in complete darkness. Brown repeats this statement (1933) but later (1935*b*) is of the opinion that the red pigment is expanded in shrimps kept in darkness.

indicated by the expanded state of the red chromatophores.[10] Yet under these two conditions, the amount of hormone in the eye-stalks is the same.

This situation is understandable if we consider that the white background reflex produces not only a maximal release of the hormone (and acceleration of synthesis due to the primary effect) but also a concomitant increase in the rate of synthesis so that there is a balance between a high rate of synthesis and a high rate of release. This balance must be attained since *Palæmonetes* retains a transparent hue for months

TABLE V

Hormone content during diurnal rhythm

Condition	*Uca pugilator*			*Uca pugnax*		
	Phase	Number of Eye-stalks Extracted	*Uca* Units/E.S.	Phase	Number of Eye-stalks Extracted	*Uca* Units/E.S.
Retina extirpated 1 week previously						
Day, light	Black	10	0.98	Black	10	0.90
Day, dark	Black	8	0.94	Black	10	1.61
Night, light	Pale	26	1.30	Inter.	18	1.00
Night, dark	Inter.*	10	0.96	Pale	8	0.91
Normal animals						
Day, light	Black	30	1.0	Black	10	0.95
Day, dark	Black	10	2.0	Black	10	0.97
Night, light	Pale	10	1.0	Inter.	18	0.93
Night, dark	Inter.	10	0.90	Pale	10	0.99
1 eye-stalk removed 3 days previously						
Day, light	Black	16	0.95			
Night, light	Pale	16	0.95			

* The abbreviation "inter." is used to designate the intermediate condition in coloration between black and pale.

if kept continually over an illuminated white background. If the white background reflex effected only a maximal release, one would reasonably expect that over a long period of white background-adaptation, the level

[10] The hormone is probably released in amounts and at a rate which constitutes a sub-minimal stimulus for the integumentary chromatophores. Some release must occur because Kleinholz (1935) writes that the retinal pigments of *Palæmonetes* are under control of the eye-stalk hormone, and in animals kept on a black background the retinal pigments are in their active state. Hence, if the same hormone affects both the retinal cells and the integumentary chromatophores, it must follow that the amount of hormone released in illuminated black-adapted animals is minimal for the eye-pigments but sub-minimal for the body pigments.

of synthesis would be outstripped by the rate of release. The glands would therefore become exhausted and the animals would become dark due to chromatophore expansion. This apparently never occurs. In the black-adapted specimens, the release of the hormone is proceeding slowly and consequently, a balance is also obtained between a lower rate of synthesis and this lower rate of release. The fact that the amount of hormone in the eye-stalks is the same in both white-adapted and black-adapted specimens is due simply to the primary effect of incident light.

This interpretation of the physiology of the eye-stalk glands may be summarized as follows: Incident light (as opposed to light reflected from backgrounds) induces an acceleration of hormone synthesis but exerts only a sub-minimal release of the hormone into the circulation. This effect of light may be called the primary effect, and occurs in specimens placed on any background provided overhead illumination is present. The white background response, however, is due to the combination of the primary effect of incident light and of the effect of reflected light, which is to produce a maximal release of the hormone and a concomitant increase in the rate of synthesis.

Uca

Uca differs from *Palæmonetes* in that it undergoes a periodic change in color and that background adaptation is lacking. Both *Uca pugilator* and *pugnax* show a periodicity of color change. The diurnal rhythm of *Uca pugnax* was described by Megašur (1912). The animals are black by day, pale by night, and this daily cycle repeats itself regardless of background or of light intensity. I have confirmed these statements and have extended them to *Uca pugilator*. When the eye-stalks are removed the rhythm is permanently abolished (at least until regeneration of the stalks takes place) and the animals remain pale regardless of background or light intensity. Periodicity is therefore controlled by a rhythmical release of the hormone into the circulation, the release occurring every 12 hours.

Several animals (*Uca pugnax*) were maintained upon an illuminated white background from Oct. 20–Nov. 14, 1935. During the day they were jet black in color, but at 5:30 P.M. they began to pale so that at 7:00 P.M. all the animals were pale (lemon yellow in color). At about 8 A.M. on the following morning the animals turned black again. The same was true of specimens maintained from Oct. 20–Nov. 14, 1935, in total darkness. Background adaptation in *Uca* seems therefore to be lacking. Periodicity is the chief factor in its color change.

Complete extirpation of both stalks leads to permanent destruction of the rhythm. Extirpation of but one eye-stalk does not impair the periodicity. One eye-stalk is therefore sufficient for the continuance of normal chromatic activity. If the retinal portion of both eye-stalks is cut off cleanly by a sharp scalpel, the rhythm is likewise interrupted.

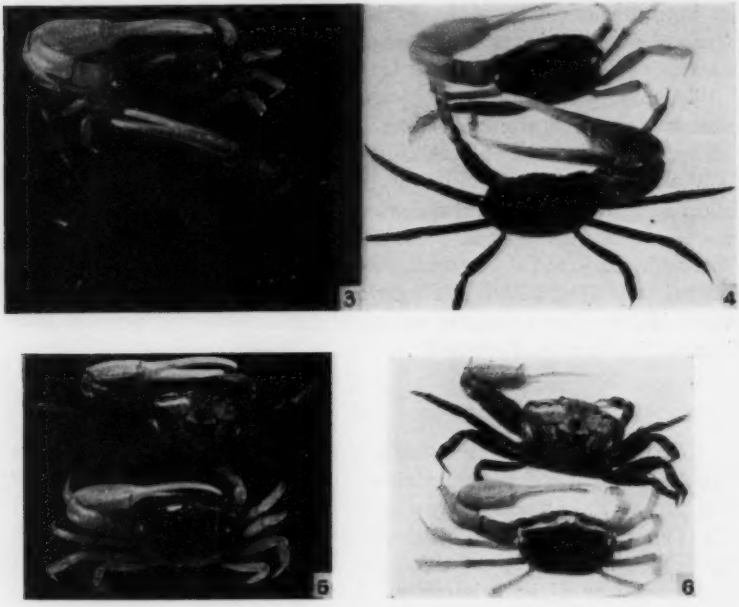

PLATE I

All photographs are from life and one-half life size.

FIG. 3. Two specimens of *Uca pugnax* photographed on a black background. Above, a specimen whose eye-stalks had been extirpated a day previously; and below, a normal animal in daylight.

FIG. 4. Same as Fig. 4, but photographed upon a white background.

FIG. 5. Two specimens of *Uca pugilator*. Above, a blinded animal 2 hours after injection of an extract of its extirpated eye-stalks. Below, an uninjected blinded specimen.

FIG. 6. Same as Fig. 6, but photographed upon a white background.

Such animals when maintained in the light on a white background remain black during both day and night, for 3 or 4 days. Similarly prepared animals maintained in total darkness remained black during both day and night for 3 days. After this period of time the rhythm appeared again but was quite erratic for a day or two, after which it appeared to

be normal again. During the past summer, I repeated some of these experiments with large numbers of animals (*Uca pugilator*). In constant light the periodic change in color occurred at 10–11 P.M. and at 9 A.M. Moreover, only about 70 per cent of the animals showed periodic changes.

The hormonal content of the stalks of selected animals under various phases of their diurnal rhythm was determined, and the results given in Table V. Animals of equal weights were used. The average weight of *Uca pugilator* was 2.5 grams, of *U. pugnax* 3.5 grams.

The results listed in Table V show that the amount of hormone present in the eye-stalks is the same whether the animals are in the pale phase of their rhythm or whether they are in the dark phase. There are some variations in the quantity of hormone extracted under different states, but these are statistically insignificant. As in *Palæmonetes,* the quantity of hormone extracted from the eye-stalks is the same in conditions during which the hormone is being continually secreted or is absent from the blood stream. This situation may be similarly interpreted. The diurnal rhythm of *Uca* is therefore an external expression of a diurnal release of the hormone into the circulation. During the 12 hours of release, the rate of production of the hormone may be increased to keep pace with its secretion into the blood.

The mechanism which controls this diurnal release differs from the release mechanism of *Palæmonetes.* The latter is definitely brought about by environmental factors (white background reflex), while in *Uca,* the diurnal release proceeds regardless of environmental factors. Furthermore, the theoretical increase in rate of synthesis is dependent on light in *Palæmonetes,* and is independent of light in *Uca,* since the diurnal rhythm proceeds in total darkness. The common feature shared by both these forms is that the release is probably controlled nervously. This is not as certain in *Uca* as it is in *Palæmonetes,* but the fact that the Blutdrüse in *Uca* are innervated and the observation that their normal physiology may be temporarily disturbed by destruction of the retinal portion of the eye-stalks seem to point towards the presence of a functional innervation. At any rate, it is difficult to imagine that the diurnal rhythm is an intrinsic property of the gland cells, for an inherent diurnal synchronism of all the cells in a gland would be somewhat surprising. Furthermore, if this conception is true, one would reasonably expect to find variations in the content of hormone during various phases of the rhythm since the glands would secrete during the day and synthesize during the night. The data in Table V show that this does not occur. It would be more satisfying to regard the blood glands as being regulated by a diurnal flow of nerve impulses. In either case, the ori-

gin of the diurnal rhythm is unknown, for one is now left without an explanation of the cause of a diurnal flow of nerve impulses. The intermediary step in the cycle is clear, however, for this is merely a diurnal release of the hormone into the circulation. The change in body coloration is but the external expression of this diurnal release.

A final experiment was performed to determine if a compensation of either hormone synthesis or release is made by the intact eye-stalk when its fellow is removed. One eye-stalk was removed from each of 32 specimens of *Uca pugilator* and 3 days later, the remaining eye-stalks were extracted and assayed. During this 3-day interval, the diurnal rhythm proceeded quite normally, the animals being maintained under illumination. The eye-stalks of 16 of these crabs were assayed during the day, the remaining 16 during the night. The results, tabulated in Table V, show that the amount of hormone in the remaining eye-stalk of such " one-eyed " crabs is the same during their dark phase as during their pale phase, and also the same, per eye-stalk, of normal crabs. Compensation, therefore, does not take place. One eye-stalk is quite sufficient to regulate chromatic activity when its fellow is removed.

Discussion

The interpretation just advanced should be considered only as a suggestion for it represents the first attempt to understand the normal physiology of the eye-stalk glands. It should also be stressed that the method of assay is sensitive enough to detect the difference between a concentration of X and one of 2X. Consequently, if slight variations do occur in the hormone content of the eye-stalks, these will not have been detected. Furthermore, inhibiting and activating systems may be present and play important parts in the biochemistry of the hormone.

It should also be emphasized that the theoretical considerations and the interpretations advanced are dependent upon the assumption that the glands in the eye-stalk are the chief if not the sole source of the manufacture of the hormone. This assumption is generally believed to be true, but certain recent observations are opposed to this notion. Hosoi (1934) found that extracts of the ventral nerve cord and male genitalia of *Penæus* produced chromatophore contraction when injected into blinded specimens of *Paratya,* showing that these extracts contained the chromatophorotropic hormone. Extracts of the stomach and muscles were also slightly active, but extracts of heart tissue were decidedly inactive. By a simple method Hosoi calculated that the eye-stalks were about one hundred times stronger in hormone content than the ventral nerve cord or genitalia. These results were criticized by

Kleinholz (1935), who attributed them to the presence of the hormone in the coagulated blood contained in these organs. Kleinholz's criticism seems to be well taken, but it is rather surprising that heart extracts were negative since these should also contain coagulated blood. At any rate, Hosoi does not describe the type of animal from which the extracts were made. If the extracts were prepared from shrimp showing the phase in which the eye-stalk hormone was being released into the circulation, Kleinholz's criticism would seem very justifiable and the importance of Hosoi's results would be uncertain. If the extracts were made from black-adapted specimens, or better, from animals whose eye-stalks were amputated some time previously, Hosoi's results could then be taken to indicate that organs other than the eye-stalks may produce the hormone. Brown (1933, 1935*a*) likewise states that extracts of the ventral nerve cord of *Palæmonetes* are active when tested on the chromatophores of blinded specimens. However, he gives no data concerning the phase of the shrimps from which his extracts were prepared nor of the potency of such extracts in relation to an extract of the eye-stalks. It would seem that Kleinholz's criticism of Hosoi's work would apply aptly to that of Brown. It has not been shown conclusively, therefore, by this type of experiment that other tissues may produce the hormone found so abundantly in the eye-stalks.

A second line of evidence bearing on this question has been advanced by Brown (1935*a*). Brown applied heat and electricity to the stubs of the eye-stalks in animals whose stalks were previously removed and observed that such stimuli induced rapid chromatophore contraction. This reaction was interpreted by him to be due to the excitation of endocrine glands of some region of the body outside of the eye-stalks by these heterologous stimuli (heat, electricity). However, this interpretation must be supported by the demonstration that the assumed endocrine glands in some region of the body are actually present, and that the contraction of the chromatophores following such stimuli is actually hormonal.

It can be concluded from this discussion that the eye-stalks are the chief source of the production of the chromatophore hormone. It can also be stated that none of the experiments already mentioned shows conclusively that tissues other than the eye-stalks may produce the hormone. The facts that under illumination blinded *Palæmonetes* remain steadily dark, and that blinded *Uca* remain continuously pale indicate that if other tissues, capable of forming the chromatophore hormone, are present they play an insignificant part in the ordinary chromatic physiology of these animals.

The interpretation of the mechanism of diurnal rhythm in *Uca* differs slightly from that advanced by Young (1935) for the cyclostome, *Lampetra*. *Uca* and *Lampetra* show many features in common. In each animal background adaptation is lacking. Each animal is pale during the night, and dark during day. Each responds to the loss of its chromatophore hormone by complete pallor, and each responds to the loss of its retinas by melanophore expansion, which is not as permanent in *Uca* as in adults of *Lampetra*. The diurnal rhythm of either *Uca* or *Lampetra* may proceed in total and enduring darkness. However, under constant illumination, the coloration of *Lampetra* remains steadily dark while the color rhythm of *Uca* may continue. To account for the periodic change in the coloration of the lamprey, Young suggested that the paired eyes in collaboration with the pineal glands affected the melanophores by a nervous inhibition of the secretion of the melanophore-expanding substance by the pituitary so that the animal becomes pale. This associates the active phase of the daily cycle with the appearance of the pale coloration of the lamprey, that is, with its nocturnal hue. The mechanism advanced for *Uca* places the active phase of the cycle with the appearance of its diurnal hue, but there appears to be no reason why Young's interpretation could not also apply to the situation in *Uca*. What actually happens in either *Uca* or *Lampetra* is the secretion of the melanophore hormone (assuming that we are dealing in terms of one melanophore substance in either case) into the circulation during the day, and its absence during the night. This daily cycle may be due to (1) a diurnal nervous stimulation of secretion, (2) a nocturnal nervous inhibition of the glands which, if not inhibited, would continue to secrete, or (3) to a combination of both. At present, it is difficult to prove which of the three possible mechanisms is correct. The interpretation that the active phase of the daily cycle in *Uca* was associated with the dark phase was made in consideration of the results obtained with *Palæmonetes*, in which the release of the hormone into the circulation is evidently due to a nervous stimulation of the glands.

In conclusion, it would be fair to outline briefly other possible explanations of the physiology of the eye-stalk glands in *Palæmonetes*. The explanation already advanced is based partly on the observations of Brown (1935*b*), which may not be entirely correct, and also takes into consideration the behavior of the retinal pigments on the basis that they are affected by the same hormone. Concerning the latter, there are no indications at present for the existence of two hormones, one for the body pigments, and one for the retinal cells. All that is known now is that the same extract which affects the body chromatophores also

affects the retinal cells (Kleinholz, 1935). The key to the whole situation lies in the behavior of the red and yellow chromatophores in animals maintained in darkness. If Brown (1935*b*, p. 320, but see also p. 328) is correct in noting that the more usual condition of the red and yellow pigments of animals maintained in darkness was slight dispersion, and that a long sojourn (2–3 weeks) in darkness resulted in the same condition of these pigments as that occurring when animals were adapted to a red background (cf. p. 319, Brown, 1935*b*), there is little reason for postulating separate autocoids for the body and retinal pigments. Threshold differences would account for the various responses. If the earlier observation of Perkins (1928) that the red and yellow pigments are contracted in darkness is correct, the existence of separate hormones would be clearly indicated (because the chief body pigments would react completely independently of the retinal pigments in darkness and on an illuminated black background), and the entire problem would be greatly simplified. Secretion and synthesis of the retinal hormone would take place only under the action of light; in darkness, release would be abolished and synthesis reduced. For the chief body pigments, it would have to be assumed that the gland produces and releases the hormone continuously, that synthesis is accelerated by incident light regardless of background, and that release is inhibited by a black background. The latter process seems theoretically difficult, for a white background reflects while a black background absorbs light. Finally, the stimulus for release or for inhibition of release may depend on the excitation of certain portions of the retina by a particular ratio of incident to reflected light. All these possibilities are well worth investigation.

Summary

A method for the standardization of the crustacean eye-stalk hormone on the blinded fiddler crab, *Uca*, has been described. The *Uca* unit has been defined as the amount of hormone contained in 1 cc. of solution, 0.05 cc. of which when injected into each of 15 specimens of *Uca pugilator* blinded 2 days previously produces a response whose average duration is about 5.0 hours. The response is measured as the amount of time intervening between the injection of the hormone and the time at which the animals again become pale, an interval during which the melanophores expand, remain expanded for some time, and finally contract. The hormone content in the eye-stalks of various crustaceans was determined. A method for the extraction and purification of the hormone has been described, and some chemical and physical properties of the hormone have been listed.

The amount of hormone extracted from the eye-stalks of *Palæmonetes* is the same regardless of whether the hormone is secreted continuously into the circulation, or whether it is continuously absent, conditions which are brought about by illuminated white and black surroundings respectively. In darkness, there is no release of the hormone into the blood, and a very low content of hormone in the eye-stalks, approximately half that obtained from the stalks of illuminated animals. It is postulated that light, regardless of background, causes an acceleration in hormone synthesis, and that light depending on certain backgrounds such as white, causes a maximal release of the hormone into the circulation with a concomitant increase in rate of production of the hormone. The diurnal color rhythm of *Uca* is an external expression of a diurnal release of the hormone into the circulation. Both release and synthesis are independent of environmental conditions, and it is suggested that they are controlled by a diurnal discharge of nerve impulses from the C.N.S. This discharge, during the day, would exert a 12-hour release of the hormone with a concomitant increased rate in production, the absence of the discharge during night would cut off release and slow down rate of synthesis.

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CALCIUM REDUCTION AND THE PROLONGATION OF LIFE IN THE EGG CELLS OF ARBACIA PUNCTULATA

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INTRODUCTION

Most investigators of the unfertilized egg cell have, naturally, been concerned with the problems of fertilization and localization. I do not believe that the egg has often been thought of as a living organism with a definite life span, quite independent of its fertilizable life. And yet, freshly shed eggs which cannot be fertilized are undoubtedly alive. Occasionally their fertilizability may be restored (Lillie, 1912). Also, aged eggs which do not respond to sperm are not necessarily dead (Woodward, 1918). In a study of longevity it is important that there be no confusion between the living condition and the ability to receive sperm and to cleave. The two are not synonymous and the test of fertilizability is not the test for life.

It seems of interest to point out that with regard to longevity the unactivated egg cell is quite different from other unicellular organisms, like the protozoans, in that its life span is very definitely limited; and, as Jacques Loeb (1913) has suggested, death of the unfertilized egg is perhaps the only case where we can be sure that it is due to "natural causes" and not to avoidable injuries. But the concept developed by Loeb and Northrop (1917) for the Metazoa, that natural death is connected with compound character, obviously falls down here where there is no organ differentiation.

This paper is concerned with the prolongation of life in the unfertilized egg cells of *Arbacia punctulata*. It seems reasonable to assume that factors which prolong life are involved also in natural death.

METHODS

Shed eggs, 98–100 per cent of which were able to cleave, were used. The eggs were washed in one or two changes of 250 cc. of filtered sea water and then concentrated into 50–60 cc. by settling. Ten-cc. samples

were added to 200 cc. of the experimental solutions in finger-bowls. These dishes, covered, were stored for the duration of an experiment on a running sea-water table in subdued daylight. The temperature for the whole series of experiments varied between 20 and 21.5° C. Old solutions were siphoned off and the concentrated eggs transferred into fresh solutions in clean dishes at least once and sometimes twice in 24 hours. As dead eggs often adhere to the bottom of the dish, an unavoidable error was introduced in calculating percentage dead at any time after the first change. However, this error does not affect the end-point when no living cells remained and it was minimized by taking readings before changing solutions.

Merck C.P. or "Reagent Grade" chemicals were used and, where obtainable, "For Biologic Work." NaCl and KCl were made up in 0.53 M concentration; $MgCl_2$ and $CaCl_2$ in 0.29 concentration.[1] NaCl, KCl and $MgCl_2$ were mixed in the proportions of $100/2\frac{1}{2}/10$ by volume, which is approximately the normal concentration of the cations in sea water. In the initial experiments (represented in Fig. 1), sea water was diluted with this Ca-free medium. Thus, to obtain $\frac{1}{6}$ calcium concentration, 500 cc. of the Ca-free medium was added to 100 cc. of sea water. The concentrations used were $\frac{2}{3}$, $\frac{1}{3}$, $\frac{1}{6}$ and $\frac{1}{12}$ calcium besides normal calcium in sea water as a control. The hydrogen ion concentrations of these solutions, measured colorimetrically, were respectively pH 8.3, 8.2–3, 7.6, 7.4 and 8.3. It is apparent that the buffering action of sea water prevented any significant change in pH down to a dilution of one part of sea water to 5 parts of Ca-free solution.

In later experiments sea water was diluted with five-sixths of artificial medium made up to contain NaCl, KCl and $MgCl_2$ in proportions as above, plus varying amounts of $CaCl_2$. Except for the controls in sea water the pH of all of these was 7.6. Solutions made up in this way were used in the experiments represented in Fig. 2.

In the experiments of Fig. 3 isosmotic dextrose (0.95 molal) was substituted for NaCl, KCl, and $MgCl_2$, and the solutions were made up with 1 part sea water, 5 parts isosmotic dextrose and sufficient isosmotic $CaCl_2$ to give the concentrations of calcium indicated. For example, to obtain $2.6 \times$ Ca the following: 150 cc. sea water, 750 cc. 0.95 molal dextrose, 91 cc. 0.29 molar $CaCl_2$.

Concentrations of calcium are calculated on the basis of 0.428 gram/liter, which is normal for sea water (Page, 1927). This point is unity on the graphs.

[1] Theoretically 0.34 M $CaCl_2$ is isotonic to sea water, but by empirical test Mr. Leon Churney of the University of Pennsylvania has found 0.29 M to be more nearly isosmotic with the *Arbacia* egg.

For observation, at least 100 eggs, usually more, were counted to obtain the percentage disintegrating. These values were plotted against time in hours and time for 50 per cent to disintegrate obtained from the curves. In some experiments samples were fertilized at each observation.

The Prolongation of Life

In a study of the effects of calcium on subsequent cytolysis in distilled water, the results of which have already been briefly reported

Table I

Effect of dilution of sea water with Ca-free medium on prolongation of life. Upper figures in horizontal rows percentage disintegrated, lower figures number of eggs counted.

Age in Hours	In Sea Water	In 2/3 Ca	In 1/3 Ca	In 1/6 Ca	In 1/12 Ca
	3.7	2.4	0	1.4	0
16½	108	125	105	146	132
	14.3	9.4	2.5	0.7	0.4
22	112	139	121	139	241
	48	48	4.4	4.6	0
39½	115	159	113	152	
	84	85	4.9	0.6	0
48	134	112	143	177	
	100	100	68	10	0.95
66¼			114	121	210
			100	74	17
90½				133	147
				100	82–100
115					218

(Schechter, 1936), it was found that exposure for several hours to Ca-free media produced no noticeable deterioration of *Arbacia* eggs and they were able to cleave when fertilized in sea water. On the other hand, 25 minutes in isosmotic $CaCl_2$ resulted in the disintegration of 40 per cent (23 out of 58 counted). After one hour and nine minutes none survived. Cleavage was reduced to zero after 15 minutes and this effect could not be reversed by an equal period of washing.

The lethal influence of pure $CaCl_2$, not shown with sodium and potassium chlorides within the time indicated above, led to the work in

which calcium concentration was varied as described under methods. To illustrate the nature of the observations the data of one experiment are presented in Tables I and II. The eggs were obtained at 2:13 P.M. August 4 from an urchin collected and brought to the laboratory at 11:10 A.M. Ninety-eight per cent cleaved on test. After washing in two changes of 250 cc. of sea water, and concentrating into 50 to 60 cc., a 10-cc. sample was placed in each of the experimental solutions at 4:30 P.M. An equal sample in sea water served as a control.

In Table I the top figure at each age is the percentage disintegrated, i.e., visibly in bad condition. The lower figure is the number of eggs upon which the percentage is based. Table II represents the percentage

TABLE II

Effect of dilution with Ca-free medium on prolongation of fertilizability in surviving eggs. Upper figures percentage cleaved, lower figures number of eggs counted.

Age in Hours at Fertilization	In Sea Water	In 2/3 Ca	In 1/3 Ca	In 1/6 Ca	In 1/12 Ca
	93	94	81	89	87
17	86	96	102	105	99
	71	50	69	61	60
22½	61	102	96	105	114
	65	50	67	59	80
40¾	62	58	116	78	84
			78	67	58
48½			101	104	127
			22	62	84
67			41	109	102
				2	45
91				51	89

which cleaved as a fraction of those which appeared to be in good condition. The lower figures are numbers of eggs upon which the percentages are based. These data exclude disintegrated eggs and show physiological condition in the survivors, insofar as fertilizability and cleavage measure such condition. Table III is derived from Tables I and II, by the product of percentage of cleavage and percentage of live eggs, and is appended to give an indication of the extension of fertilizable life in terms of the total population at the beginning.

The photographs in Plate I are taken from the above-described experiment. Photographs 1 to 4 are of eggs aged 39½ hours in sea water, ⅔ calcium, ⅓, and 1/12 calcium. They show progressively fewer disintegrated with greater lowering of the normal calcium concentration. Photographs 5 to 8 are, respectively, samples of the same, fertilized at 40¾ hours of age. Photographs 9 and 10 contrast the condition at 66¼ hours of age of unfertilized eggs in sea water (all of these having disintegrated long before) and in 1/12 calcium. The latter, fertilized when 67 hours old, show a very high percentage of cleavage (Photograph 11) and even when 91 hours old (Photograph 12) the cleavage percentage is high and early cleavage, at least, is normal.

TABLE III

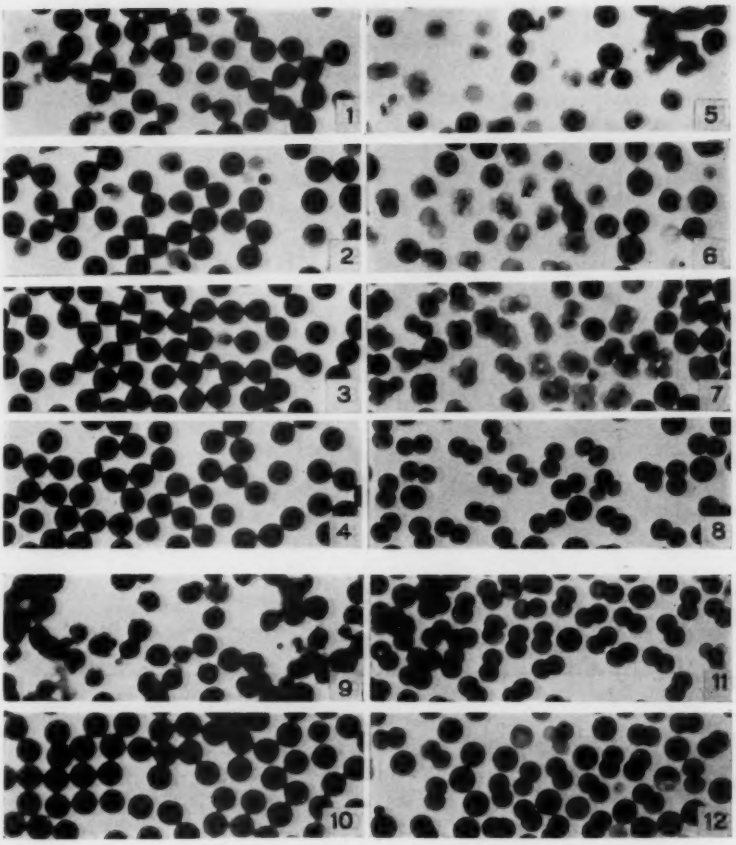
Effect of dilution with Ca-free medium. Surviving percentage of total population able to cleave.

Age in Hours at Fertilization	In Sea Water	In 2/3 Ca	In 1/3 Ca	In 1/6 Ca	In 1/12 Ca
17	89	91.8	81	87.7	87
22½	61	45.3	67.3	60.5	60
40¼	33.8	27	64	56.3	80
48½			74	66.6	58
67			7.05	56	83
91				0.52	37.4

Seven experiments of the kind just described were performed. In three the eggs of two or three females were combined, giving a total of eight and in four others single lots of eggs were used. The results of these twelve are presented in Fig. 1. Calcium concentration, in terms of that normal to sea water as unity, is plotted on the ordinate axis and time for survival of 50 per cent of the population at various concentrations on the abscissa. Full data support all points except at 1/12 calcium where measurements are available only from 5 lots of eggs.

It is apparent from the graph that the span of life increased steadily upon dilution with Ca-free medium. From 30 hours in sea water, longevity rose 300 per cent to 90 hours at 1/12 the normal calcium (and other changes in this solution to be brought out later).

Four experiments were carried out with a constant fraction of sea water, calcium variation being obtained by adding $CaCl_2$ to the artificial medium as described under methods. In this way the pH was constant and differential dilution of any possibly toxic constituents of sea water was also avoided. The results are expressed in Fig. 2, and for comparison the range from 0.88 to 0.167 calcium is shown on Fig. 1 by a dotted



EXPLANATION OF PLATE I

PHOTOGRAPHS 1–4. Eggs aged for 39½ hours in normal calcium (sea water), ⅔, ⅛, and 1/12 calcium respectively.

PHOTOGRAPHS 5–8. Cleavage in eggs fertilized after 40¾ hours in above solutions, respectively.

PHOTOGRAPH 9. Eggs aged 66¼ hours in sea water.

PHOTOGRAPH 10. Eggs aged 66¼ hours in 1/12 calcium.

PHOTOGRAPH 11. Eggs aged in 1/12 calcium, fertilized when 67 hours old.

PHOTOGRAPH 12. Eggs aged in 1/12 calcium, fertilized when 91 hours old.

line. In Fig. 2 the larger dots represent the average of observations on four lots of eggs and the smaller dots those of two. The pH for all points was 7.6 except that of sea water which was 8.3.

In Fig. 3 are given the averaged results of two experiments with $\frac{1}{6}$ sea water plus $\frac{5}{6}$ dextrose and different amounts of isosmotic $CaCl_2$ added. The measured pH varied at random from 6.7 to 7.0.

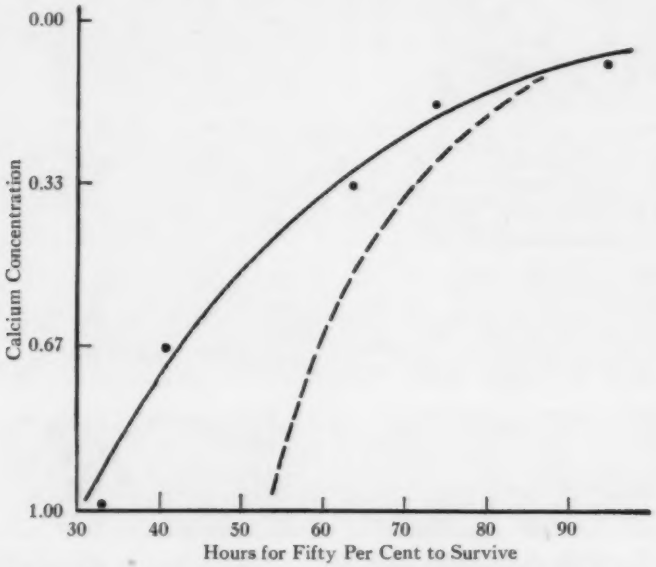


FIG. 1. Effect of dilution of sea water with isosmotic Ca-free salt solution on the longevity of unfertilized *Arbacia* egg cells. Unity on the ordinate represents undiluted sea water. pH is 8.3 except 7.6 in $\frac{1}{6}$ sea water and 7.4 in $\frac{1}{12}$.

DISCUSSION AND CONCLUSION

The results leave no doubt, I believe, that decrease in calcium prolongs the life of the *Arbacia* egg. With a step-by-step elimination of other variables this phenomenon becomes increasingly clear.

Divergence between Figs. 1 and 2 in the range of calcium concentration close to that of sea water and the fact that the control group falls off the curve on Fig. 2 may be accounted for on the basis of pH. Smith and Clowes (1924) have already shown that the optimal point for longevity is below the pH of sea water. But even with constant pH, as in Fig. 2, the effect of decreased calcium is evident. The steepness

of the curve between calcium concentrations 1 and 2.6 may mean that in this range pH becomes the limiting factor for longevity, until the

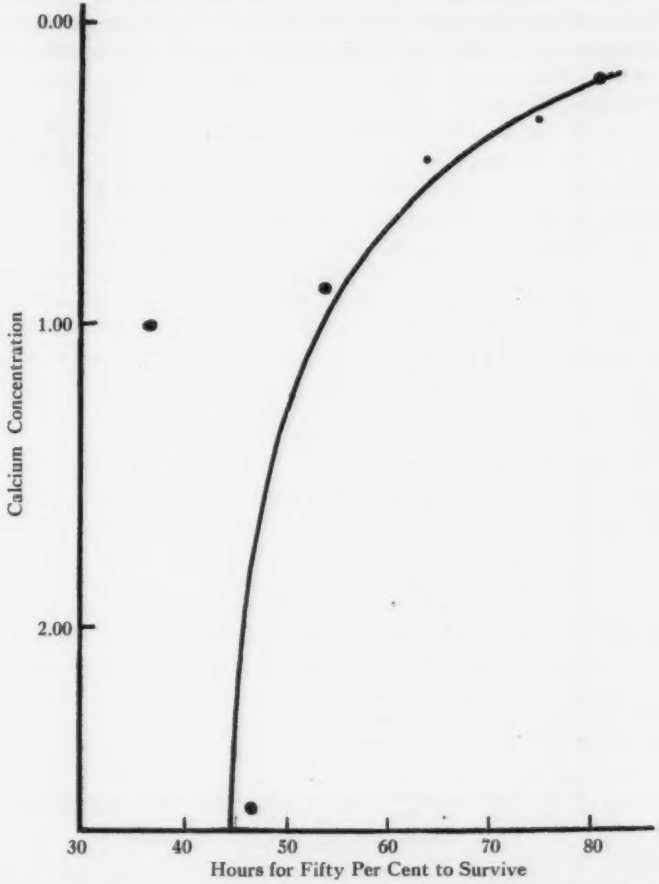


Fig. 2. Effect of calcium reduction on longevity of *Arbacia* eggs in ⅙ sea water + ⅚ Ca-free solution. pH constant at 7.6. Calcium varied by addition of 0.29 molar $CaCl_2$. Point at unity on the ordinate represents sea water control, pH 8.3.

lethal action of calcium is again sufficient to bend it toward the left. It may be that these two factors are not mutually exclusive but that acidification acts through the mechanism of calcium by a reduction in

the base-binding power of cellular materials as they approach their isoelectric points.

The use of a non-electrolyte in the experiments of Fig. 3 excludes the possibility that increase in sodium, potassium and magnesium, which was colligative with decrease in calcium in the other experiments, is re-

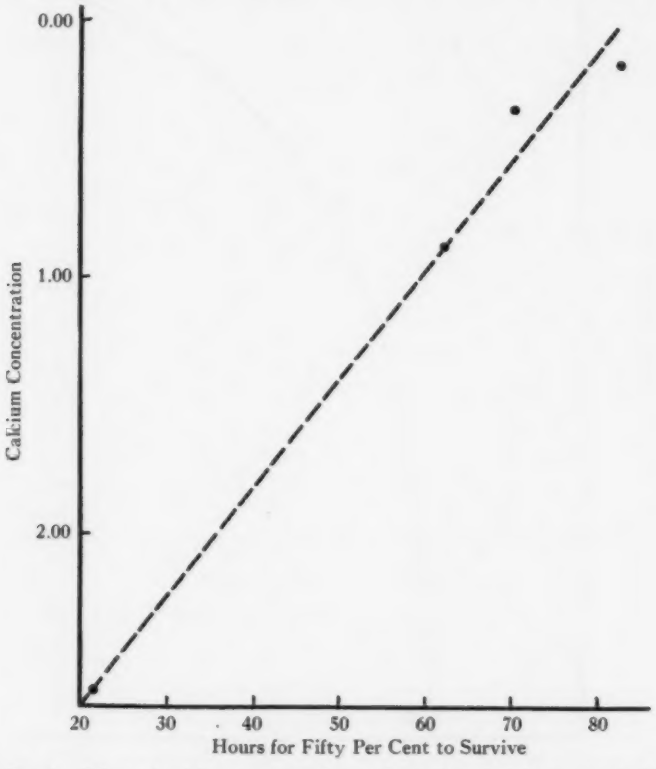


FIG. 3. Effect of calcium concentration on longevity of *Arbacia* eggs in ⅙ sea water + ⅚ 0.95 molal dextrose. pH ± 6.9. Calcium varied by addition of isosmotic $CaCl_2$.

sponsible for the results. Here there was no electrolyte variable but calcium; and it may perhaps be significant that there appears to be a most nearly straight-line relationship. Actually, however, the curve may be S-shaped, but unlike that of Fig. 2, swinging quickly toward the ordinate axis in the absence of ions antagonistic to calcium.

The influence of dextrose is, of course, a disturbing factor. In pure dextrose the permeability of the egg rises, but 0.00005 M $CaCl_2$ is sufficient to keep it at sea water level (McCutcheon and Lucké, 1928). The lowest concentration of $CaCl_2$ in my experiments with dextrose is fifty times this amount. Here, however, calcium is antagonized by sodium and potassium (in 1/6 calcium solution). At 0.34 concentration there is an excess of 0.002 M free calcium; and at this point, with permeability change presumably ruled out, life duration is 350 per cent greater than at 2.6x calcium, as seen from the graph. It must be kept in mind, however, that the permeability studies were made on fresh material exposed to the experimental solutions for short periods of time. The usefulness of the results in this discussion assumes transferability to my experimental conditions involving long exposure.

As this writing is in progress an advance abstract has appeared in which Whitaker (1936) reports that 1 per cent dextrose and 1 per cent alcohol in sea water, each prolongs the fertilizable life of *Urechis* eggs. Although it is premature to comment in the absence of a complete account, may I point out (Heilbrunn, 1934) that with low free calcium (which is probably the condition in the interior of an egg) 1 per cent alcohol (and 1 per cent ether) prevents the surface precipitation reaction, in which calcium is significantly involved. Also, Gray (1926) found that sugar and alcohols act like calcium in their stabilizing effect on the matrix of *Mytilus* gill tissue. It may, therefore, be that the action of alcohol and of sugar on the *Urechis* egg is effected through a change in the calcium equilibrium.

If calcium is a factor in determining longevity in *Arbacia* egg cells, we might expect a similar mechanism within the body of the sea urchin, where the cells may live for a prolonged period. Analysis of the cœlomic fluid [2] of female sea urchins showed a calcium content of 0.395 mg./cc. against 0.41 mg./cc. in sea water. On the basis of the data of this paper the difference, of about 3½ per cent, is insufficient to account for longevity of eggs within the animal. However, calcium content in the gonad may be lower than that of the cœlomic fluid. No direct evidence is available but Heilbrunn (1928, p. 148) has reported that eggs taken from the ovary are much more resistant to cytolysis in isosmotic $CaCl_2$ than if they are first equilibrated with the salts of sea water. Therefore, regardless of the negative results from cœlomic fluid analysis, a calcium mechanism for longevity of ovarian eggs is a possibility.

Finally, because of my interest in bioelectric phenomena (Schechter, 1934), may I point out that electrical potentials are correlated with cal-

[2] The analysis was made by Daniel Mazia of the University of Pennsylvania to whom I wish to extend my thanks.

cium changes within the range of these experiments (Dan, 1936). Electrophoretic potential rises from — 30 to — 20 millivolts as calcium concentration is reduced.

Summary

1. At ± 21° C. in sea water 50 per cent of the eggs of *Arbacia punctulata* remained undisintegrated for about thirty hours.

2. With decrease in calcium to $\frac{1}{12}$ that found in sea water and a drop in pH from 8.3 to 7.4, 50 per cent of the eggs survived for ninety hours.

3. With pH constant at 7.6, 50 per cent of the eggs survived for 53 hours in an artificial solution with normal calcium concentration and for 90 hours in $\frac{1}{12}$ calcium.

4. With other electrolytes kept constant by the use of dextrose solution 50 per cent of the eggs survived for 20 hours in 2.6x the normal calcium and for more than 80 hours in $\frac{1}{12}$ concentration.

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THE EFFECT OF ULTRA-VIOLET LIGHT UPON EARLY DEVELOPMENT IN EGGS OF URECHIS CAUPO[1]

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Introduction

During the summer of 1936 while at the Hopkins Marine Station, Pacific Grove, California, the writer observed the effect of ultra-violet radiation upon the early development in eggs of *Urechis caupo,* Fisher and MacGinitie (1928*a,* 1928*b*). The eggs of this marine worm are immature when shed, hence maturation ensues after fertilization. The present paper gives results which show the effect of ultra-violet radiation upon maturation and first cleavage, together with the effect upon the rate of these early stages. The study does not aim at a physical analysis of radiation effects, but rather, at the qualitative effects of radiation upon maturation and first cleavage.

Material and Methods

The worms were collected at Elkhorn Slough, Monterey Bay Region, California. To insure the use of gametes in best condition the usual practice was to collect animals as often as tide conditions were favorable. Experimental animals were kept no longer than three weeks. They gave a yield of eggs and sperm of high fertilization capacity throughout the entire period of the work. Quantities of gametes were removed from the worms (males and females were kept in separate aquaria) when needed. Eggs were placed immediately into dishes containing 250 cc. sea water and dry sperm were kept in covered Syracuse watch glasses.

Irradiation was by means of an Analytic Model Quartz Lamp, Hanovia, which operated on a 110-volt circuit, alternating current, 60 cycles, 5 amperes.

All handling of eggs and sperm was done at a controlled temperature of 20° C. (plus or minus 2.5° C.). For regulation of the temperature of the sea water into which the eggs were placed several water-baths were used. Within each bath a thin-walled, flat-bottomed, glass dish

[1] This work has been supported in part by a grant from the Committee on Radiation, National Research Council.

was supported. This dish held the eggs and adjacent to them was placed the bulb of a calibrated thermometer.

Conditions were standardized as much as possible. The ultra-violet lamp was allowed to burn for 10 minutes before exposures were made. During this time traces of ozone were removed and the light reached its maximum intensity. Radiations were made at a distance of 30 cm. from the source of the light and the unfertilized eggs were given non-lethal exposures to the full spectrum of the mercury arc, for periods of 30, 35, 40, 45, 50, 55, and 60 seconds.

An experiment consisted of placing eggs whose controls gave 99 per cent to 100 per cent polar body extrusion and 97 per cent to 100 per cent first cleavage, in a thin layer on the bottom of a dish filled with sea water to a depth of 2.5 mm. This dish was placed in the constant temperature bath under the ultra-violet light and the eggs were irradiated. The dish was then removed and placed in a constant temperature bath fitted to the microscope, where the eggs were observed for five minutes for activation (development of fertilization membranes) by the radiation. When no activation was found the eggs were inseminated with a standard sperm suspension (one drop of dry sperm from a capillary pipette to five cc. sea water) and allowed to develop. The eggs were examined later for the extrusion of polar bodies and for first cleavage. Counts were made of the number of eggs in 100 which extruded first and second polar bodies, and of the number of eggs in 100 which showed first cleavage. These counts were repeated 19 times for each group of irradiated eggs. Twelve sets of experiments were made so that the data given below are for 24,000 eggs at each exposure.

The time-lapse was measured also, from the time of the insemination of the eggs to the time of the beginning of the particular stage considered. The time-lapse measurements for polar body extrusion were made on the reacting half of 10 eggs in the field of the microscope at the time of observation and the criterion was that moment when the polar body was sufficiently extruded to be identified unmistakably at the periphery of the egg. In cases where eggs were irradiated for 50, 55, or 60 seconds it was not possible to measure the time-lapse for the reacting half of 10 eggs because of the small number of polar bodies which could be found at the periphery of the eggs. I therefore measured the time-lapse for individual eggs and considered the average of the measurements made in the 20 counts as the time-lapse measurement. The criterion for first cleavage was that moment when the egg was elongated unmistakably at or in one or more axes (according to the regularity of cleavage) and the cleavage furrow could be seen as a thin, shining line which was especially clearly defined with the type of

illumination used. The time-lapse was measured for the reacting half of 10 eggs in the field of the microscope, but at exposures of 50, 55, or 60 seconds when there were very few cleaving eggs it was necessary to measure the time-lapse for individual eggs and to take the average of the measurements made in the 20 counts as the time-lapse from insemination to first cleavage.

The Experiments

The extrusion of polar bodies and the first cleavage of the egg, together with the time after insemination at which these phenomena occur, are affected when the egg is irradiated before fertilization. The action

Table I

Percentage of polar body extrusion and first cleavage in eggs of *Urechis caupo* exposed to ultra-violet light for various lengths of time at a distance of 30 centimeters.

Length of Exposure (seconds)	First Polar Bodies	Second Polar Bodies	First Cleavage
30	91.3	88.9	73.1
35	78.4	73.9	57.7
40	63.8	55.1	40.7
45	31.4	18.5	14.0
50	9.5	2.8	5.2
55	4.7	1.4	4.2
60	2.3	0.68	2.5

All controls showed 99 per cent to 100 per cent polar body extrusion and 97 per cent to 100 per cent first cleavage.

The percentage is based upon the average of 12 experiments in each of which 2,000 eggs were counted.

of ultra-violet light upon these early stages in development is reported as the percentage of extrusion of polar bodies and cleavage, and the effect upon the rate of reaction is reported as the percentage increase in the time-lapse for each stage. The results are presented.

Percentage Extrusion of Polar Bodies and Percentage Cleavage in Irradiated Eggs

From the results shown in Table I it is apparent that extrusion of polar bodies and the first cleavage of the egg are suppressed when eggs are irradiated with ultra-violet light. Different exposures varying from 30 to 60 seconds, with an increase of five seconds for successive durations of exposure, produce increasing suppression of the developmental stages until the longer periods of exposure are reached when there is a practically total suppression. Average percentages for all irradiations are presented for each duration of exposure which show unmistakably

that the suppression of polar body extrusion and of first cleavage varies directly with the length of exposure. Since the data are based upon observations and counts of large numbers of eggs (the average percentages are for 24,000 eggs) the evidence is fairly conclusive. These results on the suppression of polar body extrusion confirm observations

TABLE II

Effect of ultra-violet light upon the time-lapse from insemination to polar body extrusion and to first cleavage in eggs of *Urechis caupo* exposed for various lengths of time at a distance of 30 cm. Percentage increase in time-lapse equals $\frac{\text{time-lapse in radiated eggs—time-lapse in control}}{\text{time-lapse in control}} \times 100.$

Stage	Length of Exposure (seconds)	Time-lapse in Radiated Eggs (minutes)	Time-lapse in Control (minutes)	Per Cent Increase in Time-lapse
First polar bodies	30	30.0	29.8	0.67
	35	31.2	28.5	9.47
	40	32.1	28.3	13.43
	45	34.8	28.6	21.68
	50	38.3	27.5	39.27
	55	41.3	28.3	45.94
	60	42.3	28.6	47.90
Second polar bodies	30	44.3	42.3	4.73
	35	46.4	42.6	8.92
	40	47.3	42.3	11.82
	45	52.9	43.8	20.78
	50	55.6	42.1	32.07
	55	57.7	42.7	35.10
	60	59.7	42.2	40.76
First cleavage	30	69.2	67.4	2.67
	35	70.1	67.2	4.31
	40	70.3	68.0	3.38
	45	75.2	68.9	9.14
	50	82.5	69.2	19.22
	55	84.0	68.7	22.27
	60	89.2	68.5	30.22

Each time-lapse measurement represents the average time from insemination to the particular stage and is based upon data for the reacting half of 24,000 eggs observed at each exposure save 50, 55, and 60 seconds. At these exposures the average time-lapse was taken for the number of eggs in 24,000 which showed the particular stage.

of Just (1933) for eggs of *Nereis limbata*. From the protocols of this observer the evidence shows that radiation effects were so pronounced that essentially a total suppression of polar body extrusion was caused when eggs were irradiated for 60 seconds at a distance of 25.5 cm. from the lamp. Just reported similar results when eggs were given longer exposures at different distances from the lamp.

Percentage of Increase in Time-lapse from Insemination to Polar Body Extrusion and First Cleavage

The data in Table II show the effect of ultra-violet radiation upon the rate of the early developmental stages in eggs of *Urechis caupo*. Time-lapse measurements for eggs irradiated at different exposures were compared with similar measurements in controls and the relation between the time-lapse from insemination to a particular stage in both the experimental and the control eggs was given as the percentage increase in time-lapse. The data show that wherever a developmental stage was affected by radiation the reaction time of the egg (time-lapse from insemination to the stage) was retarded. At the comparatively short lengths of exposure the percentage increase in the time-lapse was small. Each increase in length of exposure caused an increase in the percentage increase in time-lapse. The steady increase in the time-lapse from insemination of the egg to a particular stage as indicated in Table II may be regarded as evidence that irradiation of eggs not only affects certain stages in development but also affects the reaction rate of the eggs.

Discussion

Suppression of polar bodies and of cleavage by such agencies as exposure to extremes of the viable range of temperature for eggs, treatment with hypotonic sea water, or subjection to the action of narcotizing substances, before or after activation, has been reported by many investigators. The data reported here suggest ultra-violet light as a most effective agent for suppression of early developmental stages.

The exact nature of the action of ultra-violet radiation is not definitely known. An important factor appears to be the extent of the absorption of radiant energy by the inner protoplasm of the egg and by the superficial layer, each of which is a site of complex reactions which underlie morphological changes and developmental processes. Evidence presented by Redfield and Bright (1921) and Just (1933) shows an alteration of the initial changes involved in the cortical reaction in the egg of *Nereis* fertilized after exposure to ultra-violet light. Another type of evidence is given by Tchahotine (1921*a*), who correlated local centers of injury produced in the peripheral layer of sea urchin eggs with permeability changes. Tchahotine (1921*b*) further pointed out the probability of the coagulation of the colloids of the superficial layer of the irradiated sea-urchin egg. While eggs of *Urechis caupo*, unlike eggs of *Nereis limbata*, extrude no jelly following fertilization and show no visible alteration of the superficial layer other than the separation of a tough, pellicle-like membrane (see Chase, 1935) from the vitellus of

the egg which develops into the fertilization membrane, it is possible that certain changes take place in the egg cortex which are affected by radiation as in the case of other species of eggs. A serious alteration of the physical and chemical properties of the peripheral layer of the eggs conceivably may be a factor in the suppression of polar body extrusion in eggs which are radiated before they are fertilized.

On the other hand, the developmental stages which have been studied are closely related to various phenomena which occur deep within the egg. Of these the viscosity changes are the most widely studied. While no attempt was made to observe the effect of radiation on such changes in eggs of *Urechis caupo*, evidences of the effect of ultra-violet radiation upon viscosity of protoplasm of other eggs is reported. Of these investigations the most significant for this particular problem are the observations on the egg of *Ascaris*, which falls in the same category with the egg of *Urechis* with respect to the time at which the egg may be fertilized. Schleip (1923), in the course of observations on the effect of ultra-violet radiation on morphological components of *Ascaris* eggs, reported increased viscosity. Similarly, Ruppert (1924) centrifuged radiated eggs in his studies on the effect of ultra-violet light upon different stages in the development of eggs of *Ascaris* and his results indicate increased viscosity. Such changes alone may be associated with marked inhibition of phenomena which underlie maturation and cleavage processes and the resulting suppression of these stages in development.

A cytological study of the irradiated eggs is being made and it may give significant evidence on the effect of ultra-violet light upon morphological changes in the eggs.

Summary

1. When unfertilized eggs of *Urechis caupo* are exposed to ultra-violet light for different lengths of time, then fertilized, polar body extrusion and first cleavage are suppressed. In addition to the suppression of these stages radiation causes an increase in the reaction rate of the egg which bears a direct relation to the length of exposure.

2. Possible factors in the suppression of these stages in maturation and first cleavage are the alteration or probable injury of the egg cortex and accompanying changes in its chemical and physical properties, and viscosity changes in the egg endoplasm which inhibit internal phenomena.

The writer wishes to express his gratitude to Dr. A. C. Giese and Mr. E. W. Lowrance, School of Biological Sciences, Stanford University, and Professor

Frank Coleman, Department of Physics, Howard University, for technical advice and assistance. He is also indebted to Dr. W. K. Fisher and the staff at Hopkins Marine Station for their many courtesies.

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THE ELECTRICAL CHARGE ON NUCLEAR CONSTITUENTS (SALIVARY GLAND CELLS OF SCIARA COPROPHILA)

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In spite of the importance of electric charge in the behavior of colloidal materials, very little work has been done to determine either the sign or the magnitude of the charge on the particles of the cell nucleus. It is possible by cataphoretic experiments to determine at least the sign of the charge on the various structural elements of the nucleus. At the present time there is almost no direct data on the sign of the electric charge on animal chromosomes or on the nuclei of living animal cells. Botta is one of the few investigators who has attacked the problem. He reports that the chromosomes of embryonic chick heart cells are negatively charged during mitosis, but that there is little or no effect of the electric current on the chromatin during interkinesis. von Lehotzky states that he was unable to obtain results in his experiments on Protozoa and on frog blood cells.

It is possible to obtain definite information concerning the sign of the charge on giant chromosomes such as are found in the salivary glands of fly larvæ. The glands of the dipteran *Sciara coprophila* are especially favorable material for cataphoretic studies. The glands were dissected out of the larvae and studied in the Ringer's solution used by Bělař for grasshopper spermatocytes. The isotonicity of this solution with the salivary gland cells was determined by comparing glands in body fluid with those in the Ringer's solution.

The electrical set-up used in these studies consisted of a non-polarizable system of Cu–$CuSO_4$ electrodes with agar bridges. The lower extremities of two tubes, 6 mm. in diameter, were filled with a 3 per cent agar gel made up in Ringer's solution; above this gel was placed a saturated solution of $CuSO_4$, into which the ends of the copper wires carrying the current were introduced. A double-throw switch in the circuit permitted the reversal of the direction of current flow. A gland, dissected out in Ringer's solution, was placed in a drop of this medium on a glass slide, and a cover-glass was supported above it. The agar tips of the electrodes were placed in contact with the fluid, at oppo-

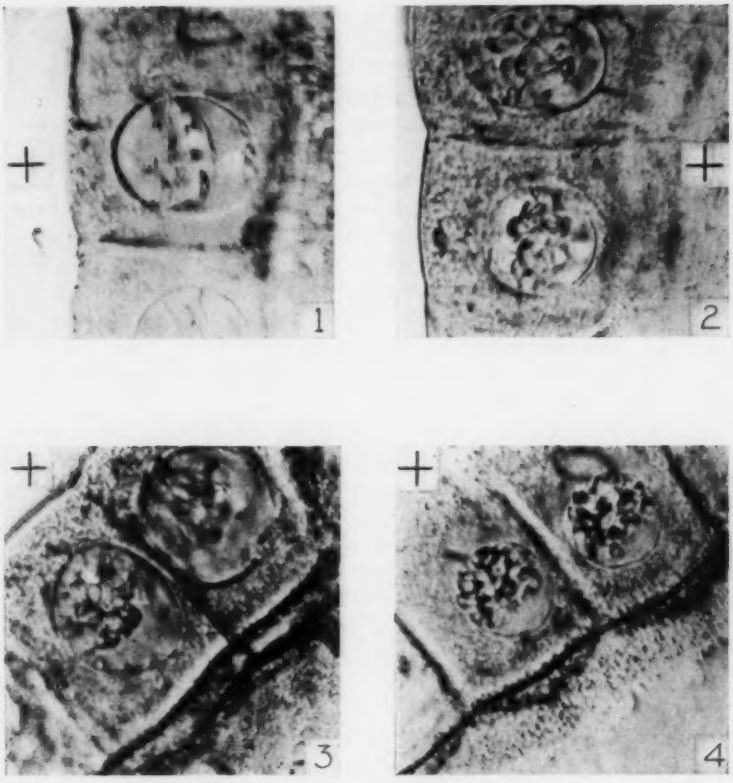
[1] We are pleased to acknowledge the aid of Dr. L. V. Heilbrunn of the University of Pennsylvania during the course of this investigation. We are also grateful to Dr. C. W. Metz of Johns Hopkins University for furnishing us with cultures of *Sciara coprophila*.

site ends of the slide. By varying the amount of fluid between the electrodes the current strength was varied from approximately 5 to 25 milliamperes. The strength of the current passing through the nucleus was not, of course, determined.

In the course of these experiments, one of the difficulties which presented itself was that of ascertaining whether or not the cell was alive. We believe that the nuclei and the chromosomes contained within them were alive, at least during their first responses to the passage of the current. In any case, all subsequent reactions were essentially the same, even in cases where there was reason to suspect that the cell was dead. After the current had passed through the cell for a short time, apparently irreversible cytosomal coagulation set in, indicating the death of the cell. In cases of extreme coagulation, very little, if any, response to the passage of the current was noted on the part of either the nuclei as a whole or the chromosomes.

In order that the chromosomes migrate cataphoretically, it is essential that the electric current pass through the gland cell and through the nucleus. Conditions must, therefore, favor such a passage of current. If the gland is placed in a large quantity of salt solution, practically all the electric current will pass around the cells. However, as the quantity of surrounding fluid, or its conductance, is decreased, the current will begin to flow through the cells. In the case of the salivary glands, the position of the gland in relation to the direction of current flow is of primary importance. If the long, spindle-shaped gland is parallel to the lines of current flow, the current will tend to flow along the central canal of the gland. On the other hand, if the gland is perpendicular to the lines of current flow, there is much more tendency for the current to pass through the cells, partly because of the fact that in this position the canal does not act as a short circuit, but also because of the fact that the path of the current leads through only two cells, and not through a long line of them.

The first apparent effects of the current flow on the nucleus were usually an increase in the nuclear volume, and an increase in the refractivity of the chromosomes. The nucleus as a whole, i.e., the nuclear membrane, moved toward the cathode (positive charge), whereas the chromosomes contained within it moved toward the anode (negative charge). These two opposing movements are simultaneous (see Fig. 4). When the direction of current flow was reversed, both the nuclei and the chromosomes responded instantaneously to the change by a corresponding reversal of their migration (see Figs. 1, 2 and 3). This reversal of migration was obtainable several times before the death of the cell and the subsequent coagulation prevented any response. When the circuit was broken, the nuclei and the chromosomes tended to return to



EXPLANATION OF PLATE I

Photomicrographs of unfixed and unstained cells of the salivary glands of *Sciara coprophila* larvæ, showing the effect of the passage of electrical currents. The anode is indicated, in each case, by a + sign. Figures 1, 2, and 3 are the same cells under different experimental conditions. Figure 4 shows cells of another preparation.

FIG. 1. Chromosomes migrating toward the anode, nuclear membrane toward the cathode. Note the crenation of the nuclear membrane as it is pressed against the cytosomal granules. Eight milliamperes.

FIG. 2. Same, direction of the current reversed. Chromosomes migrating toward anode. Eight milliamperes.

FIG. 3. Same, direction of current again reversed, establishing same polarity as in 1. Eight milliamperes.

FIG. 4. Another preparation. The nucleus as a whole is moving toward the cathode; its former position in the cytosome is indicated by the clear area toward the anode. The chromosomes are shown oriented toward the anode. Ten milliamperes.

their original positions in the cell. This was due, presumably, to a polarizing current in the reverse direction.

The movement toward the cathode and the simultaneous movement of the chromosomes toward the anode brought about distortions and abnormalities of the nuclear membrane (see Fig. 1). The chromosomes, in their movement toward the positive pole, often exerted sufficient pressure against the nuclear membrane to bring about an elongation of the nucleus in the direction of the current flow. On the other hand, the opposing force with which the nucleus as a whole moved toward the cathode was at times great enough to carry the chromosomes with it toward this pole (see Fig. 4). In such cases, however, the chromosomes always remained on the anodal side of the nucleus.

The nuclei, in their movement toward the cathode, tended to resume their spherical shape until the opposing motion of the chromosomes made this impossible, as described above, or until their movement toward the cathode was prevented by the densely packed granules of the cytosome. In the latter case, the nuclei often flattened themselves against these granules, while the anodal side of the nuclear membrane continued to flow inward (toward the cathode), carrying the chromosomes with it. This pressure of the membrane against the chromosomes often brought about a crenation of the membrane. Upon the breaking of the circuit, the nuclei tended to return to their former positions in the cells, and in doing so, tended to resume their spherical shape.

In cells which were mechanically crushed, nuclei often remained intact and floated partially or entirely free of the cytoplasm. Nuclei which were altogether free to move through the medium migrated toward the cathode. Since these nuclei were approximately midway between the slide and the cover-glass, their migration to the negative pole could not be due to the water current of endosmosis that flows in the opposite direction through the center of the chamber. The chromosomes, in these cases, were attracted to the anodal side of the nuclei, but with a force generally insufficient to distort the nuclei or to deter their rapid motion toward the cathode. Nuclei partially free from the cytoplasm, but adhering to the latter, displayed the characteristic reactions described in preceding paragraphs.

The observations indicate that the charge on the chromosomes is negative. This is in accord with various studies on plant chromatin (Pentemalli, McClendon, Hardy, Meier, Zeidler, von Lehotzky). Most of the observations of these investigators were made on material fixed after the exposure of the material to electric current, and such a procedure is perhaps open to question. However, von Lehotzky studied living plant cells.

The observation that the nucleus moves toward the cathode is new.

Perhaps other observers failed to note such a movement because of the fact that for the most part their observations were made on material fixed presumably after the cessation of flow of the current. In our own studies we observed that as soon as the circuit was broken the nuclei immediately tended to return to their original positions.

The positive charge on the nucleus as a whole is perhaps conditioned by a positive charge on the cytoplasmic colloids. Just as a quartz particle or a blood cell takes its charge from the colloids in which it is immersed and which are adsorbed on its surface, so the nucleus may owe its positive charge to the charge of protoplasmic colloids (in this connection compare Heilbrunn, 1928). The fact that isolated nuclei are positively charged does not necessarily invalidate this concept.

These experiments give us an indication of the complex electrical character of the cell components, in contrast to the excessive simplicity revealed in the cataphoresis of quartz particles or other inanimate systems. We have a living cell with a negative charge at the surface, perhaps a positive charge in the cytosome, a positive charge on the nucleus imbedded in it, and a negative charge within the nucleus (on the chromosomes).

In conclusion, this paper has attempted to stress the fact that the nucleus as a whole is positively charged, while the chromosomes within it are negatively charged. The complexity of the living system with respect to the electrical charge of the cell components has been contrasted with the apparent simplicity of inanimate systems. A more quantitative study of these phenomena is planned.

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DEVELOPMENT OF THE PRIMARY GONAD AND SEXUAL PHASES IN VENUS MERCENARIA LINNÆUS[1]

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Introduction

Sexuality of Pelecypoda has been a subject of scientific study since the end of the seventeenth century. A review of the literature on this subject reveals that in some of the forms that have been carefully studied many individuals exhibit at least one change of functional sexuality during their lifetime (Roughley, 1933; Coe, 1936*a*, 1936*b*). As a rule, a strong tendency toward protandry has been observed. Thus far, however, only two genera of Pelecypoda, namely, *Ostrea* and *Teredo*, have been extensively studied. It is true that these studies have contributed greatly to our knowledge of sex ratios and sexual phases in these bivalves, but it also seems possible that a thorough study of a representative species belonging to a genus widely differing in its mode of living from oysters or *Teredo* might furnish additional information leading to a better understanding of the sexual phenomena of mollusks. *Venus mercenaria*, the hard-shelled clam of the Altantic Coast commonly known as "quohog," was selected for this study.

This proved to be a fortunate choice, for the species was found to pass through phases of sexuality somewhat differing from those previously described for any member of the group. Like the teredos and the oysters, the hard-shelled clam shows a marked degree of protandry but there is no evidence of a second change of sexuality. The primary gonad is more or less distinctly bisexual but in nearly all individuals it becomes differentiated into a spermary when the animal is only a few millimeters in length and only a few months of age. The young individual may function as a male at the end of its first summer, retaining this phase of sexuality during the winter and becoming fully functional at the age of about one year. Others first become functional in their second summer.

About half of all the young clams examined retained the male phase

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of sexuality after the discharge of the spermatozoa, while an approximately equal number transformed to females. The sex ratios during the second winter and thereafter are thus equalized.

The writer desires to express his appreciation to Professor W. R. Coe for his advice and criticism throughout the course of this work.

Material and Methods

V. mercenaria is found along the Atlantic Coast from the Gulf of St. Lawrence to the coast of Texas. In the past few decades this species has become established on the Pacific Coast where it was transplanted with the eastern oyster.

The material generally used in this work was collected in Long Island Sound, near Milford, Connecticut. Young clams were obtained at regular intervals from a single clam bed and prepared for microscopic examination. A large number of small clams was also taken from concrete tide-filling tanks with a capacity of several thousand gallons, at the U. S. Fisheries Biological Laboratory in Milford, Connecticut, where environmental conditions, such as temperature and salinity of the water were observed and recorded. In the preparation of the material for study, standard methods of cytology and histology were employed.

In the present study all the observations on living material were checked by histologically prepared material in order to determine accurately the characteristics of the constituent cells of the gonads. The method employed by Spärck (1925), which consisted of boring a small hole through the shell of an animal and removing a piece of the gonad for examination, is not satisfactory, as very misleading conclusions may be reached by assuming the condition of the entire reproductive system from the examination of a single small sample of the gonad. Similarly, the method of Orton (1927), consisting largely of the examination of the living tissue to note the condition of the gonad with regard to the production of sex elements, is also open to criticism because of the impossibility of distinguishing many important details under such conditions.

Age and Size

A review of the literature fails to disclose any important contributions on the subject of sexuality of *V. mercenaria* in general and on the development of the primary gonads in particular. The only work is that of Belding (1912), where he states that the average hard-shelled clam is capable of spawning when in its third summer, for sexual products

could not be found at an earlier age. The sexes were found to be separate, each animal presumably remaining either male or female all its life. He gives no histological data, and it is apparent from his work that his conclusions are based only on macroscopic examination of living clams and upon his observations of their spawning activities.

It was impossible to obtain for this study small clams whose shell-length was less than 4 mm. long. Therefore the smallest animals studied were 4–5 mm. long. These were found at Trumbull Beach, Long Island Sound, during September and October. As the age of studied animals is of importance, several attempts were made to secure clams of a known age. This was accomplished by collecting seed clams and by keeping them under observation. There are two possible conclusions as to the age of small, 5–7 mm. clams, collected for this study during October–December; namely, that they set in the year they were collected, in which case their age would have been only a few months, or that they were about 14–18 months old, setting the preceding year. Belding (1912), studying *V. mercenaria* along the coast of Massachusetts over a period of five years, found that the rate of growth of the clam is largely determined by its environment, and that, as a rule, the growth in any bed is fairly uniform. In his experiments at Monomoy Point, he found that the average size of a 14-months-old clam (collected in October) was 25.59 mm. Judging by the description of the Monomoy Point experiment, the environmental conditions there closely resemble those of Trumbull Beach, at which place the young clams were obtained for the present work. The similarity of conditions of the two places makes it logical to assume that the rate of growth of clams at Trumbull Beach is more or less identical to that of clams of Monomoy Point. Thus, clams 5–7 mm. long, collected in October–December, presumably were of that, and not of the preceding year set. This conclusion is supported by the observation of Belding (1912) that young clams immediately after setting showed an average gain of 3.4 mm. per month, which would make a length of about 10 mm. by the end of November.

In the present paper all the remarks concerning the size of animals refer to the length of the shell.

Development of Primary Bisexual Gonad

Gonad tissue first appearing in the young animal consists of a very thin layer of cells between the muscular body wall and the stomach. Gonads of the juvenile phase first appear, not near the muscular body walls, but a short distance from them at the level of the heart or slightly below it. The follicles of the juvenile gonad are at first composed of a single layer of germinal epithelium cells. These cells of elongated type

are rather irregular in shape and size and possess very large, deeply-staining nuclei. During the early stages there is virtually no lumen

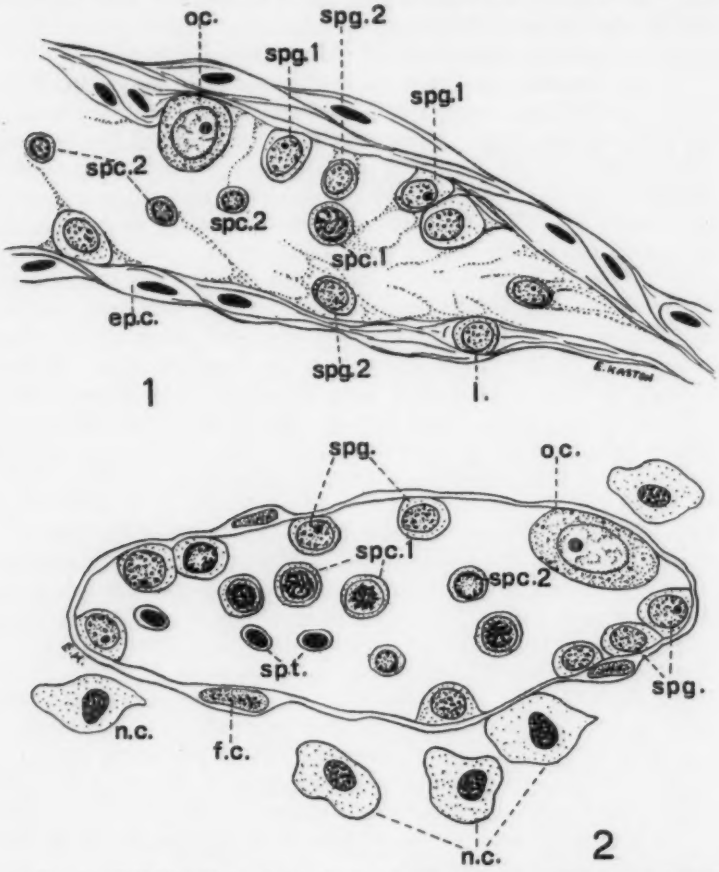


FIG. 1. Primitive bisexual gonad of a young clam soon after formation of the lumen. *I*, indifferent cell; *ep.c.*, follicular cells; *oc.*, young ovocyte; *spg.1*, primary spermatogonia; *spg.2*, secondary spermatogonia; *spc.1*, primary spermatocytes; *spc.2*, secondary spermatocytes.

FIG. 2. Primary bisexual gonad in a young clam (4–6 mm.) showing large ovocyte (*oc.*), spermatogonia (*spg.*), primary spermatocytes (*spc.1*), secondary spermatocytes (*spc.2*), and spermatids (*spt.*); *f.c.*, follicular cells. Nutritive cells (*n.c.*) are seen near the follicular wall.

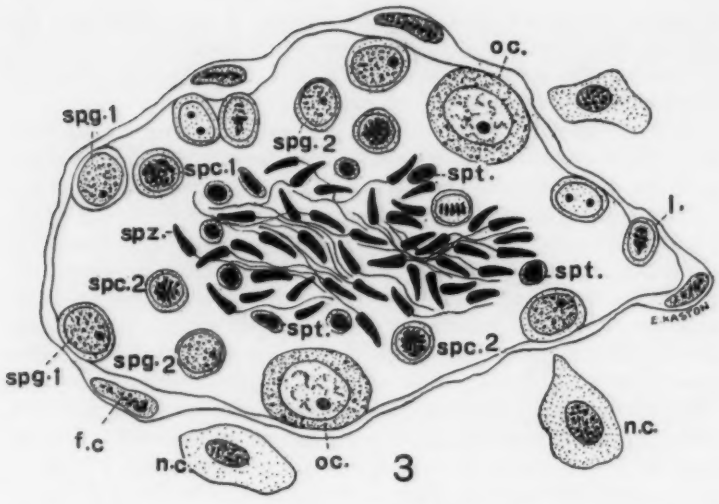
formed, the walls of the follicles being almost in contact with each other. As the animal grows, the follicles of the primary gonad begin

to ramify through the loose connective tissue. A few weeks later the germinal epithelium begins to differentiate into gonia and at this time the lumen in the follicle is formed (Fig. 1). Rapid proliferation and specialization of cells follows.

In small animals, 5–12 mm. long, the gonad does not extend very far in the ventral direction, seldom reaching below the line corresponding to the middle part of the stomach. The follicles of the gonad are not very numerous, and usually only six to eight of them can be found in one cross-section of the entire animal. In cross-sections the gonad follicles of such animals already show the lumen, which is usually oval or round in shape with a greatest diameter of 50–100 microns. Careful study of sections reveals that different gonad follicles of the same animal exhibit a widely different degree of development and bisexuality. Some of the follicles may consist of only a few indifferent cells while in others the germinal epithelium has already differentiated into male and female cells. All degrees of such processes can be observed. In some cases the follicle contains a few gonia along its wall and a mass of spermatogenic cells in early stages of development in the lumen (Fig. 2). In other, more advanced, cases spermatozoa are already formed, sometimes occupying the largest part of the lumen, while a few small ovocytes showing mitochondrial filaments of yolk nuclei are found along the walls of the follicle (Fig. 3). Gonads at such stages are distinctly bisexual and because the proliferation of spermatogenic cells is very rapid such young gonads acquire a strong male appearance (Loosanoff, 1936*a*).

In some cases the appearance of the follicles looks as though the spermatozoa were already discharged. For instance, a large sample of animals 5–7 mm. long was collected on Trumbull Beach in October, 1934. Upon examination of the prepared material it was observed that some of the follicles had already discharged spermatozoa. The lumen of such follicles was large and empty while along its walls young ovocytes and indifferent cells were present. As a rule, few phagocytes could be found inside the lumen (Fig. 4). With the purpose in view of establishing the age of those animals, very careful examination of their shells was made under a dissecting microscope. In not a single case was an indication of the winter ring found. The outer surfaces showed an embryonic shell and 7–13 growth lines formed at approximately equal intervals (Fig. 5). Thus the studies of the shells indicated that the animals of that sample were only a few months old.

If the evidence that *V. mercenaria* produces and discharges gametes during the first summer of its life is reliable, then it resembles in this respect many of the other bivalves whose sexual development is well



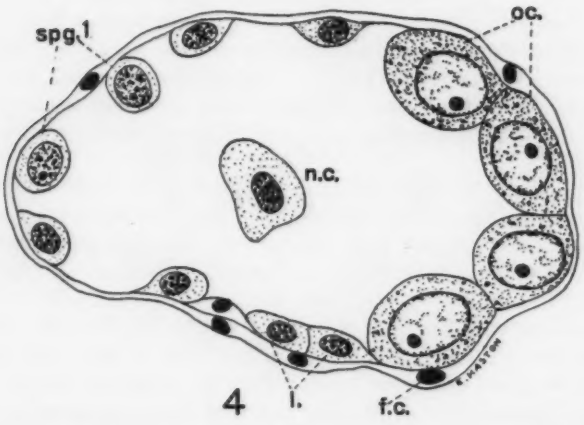


FIG. 3. Primary bisexual gonad of predominantly male character. Spermatozoa are present, occupying the center of the lumen. A few ovocytes (*oc.*) are lying along the follicular wall. *l*, indifferent cells; *spg.1*, primary spermatogonia; *spg.2*, secondary spermatogonia; *spc.1*, primary spermatocytes; *spc.2*, secondary spermatocytes; *spt.*, spermatids in different stages of spermioteleosis; *spz.*, spermatozoa; *n.c.*, nutritive cells; *f.c.*, follicle cells. Size 7–12 mm.

FIG. 4. Gonad follicle of a young clam (7 mm.) after discharge of the first crop of spermatozoa. *l*, indifferent cells; *oc.*, young ovocytes; *spg.1*, primary spermatogonia; *n.c.*, phagocyte; *f.c.*, follicle cells.

known (Nelson, 1922; Coe, 1936*c*). It is apparent, however, that there cannot be a definite rule applied to all Pelecypoda mollusks as to the time when they first form functional gametes. As a matter of fact, considerable differences are often found in closely related species and in different localities, as Spärck (1925) has so clearly demonstrated in *O. edulis*. *O. lurida* is capable of passing through three sexual phases during the first year of development in warm waters (Coe, 1932), while *O. virginica* of the northern part of the eastern coast of the United States does not form any ripe gametes until the second year (Needler, 1932). In *V. mercenaria,* ripe spermatozoa are formed within 3–5 months after setting. Among several hundred young animals studied, approximately 98 per cent of them passed through such a protandric male phase. The remaining 2 per cent appeared to develop into females without passing through a functional male phase.

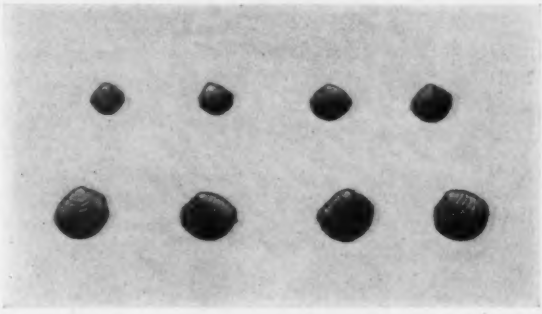


FIG. 5. Shells of the young clams, age about 3 to 4 months, collected in October–November, showing the absence of the winter ring. All of these were already provided with gonads containing mature spermatozoa. Natural size.

CONDITION DURING FIRST WINTER

According to the writer's observations, the shell-growth of clams in Long Island Sound ceases in November. Small (6–10 mm.) clams examined at that time differ very little from those studied in October. Again gonad follicles in various stages of development are found. In many follicles spermatogenesis continues and cells of all stages of development, including spermatozoa, are present (Fig. 6). In other cases spermatozoa have already been discharged and the distended lumen is virtually empty (Fig. 4). Often numerous phagocytes are seen invading the lumen or attached to the walls of the follicle. Those presumably ingest the residual spermatic cells. In a few instances the gonad tissue has already ramified ventrally below the stomach and individual follicles

can be seen confined to the space between the muscular body wall and the digestive gland.

By the middle part of December the temperature of the water of Long Island Sound reaches the point at which the period of hibernation for this species begins (Loosanoff, 1936*b*). Examination of young animals collected during that period, which extends until the middle part of April, or until the water temperature rises above 5.0° C., reveals that

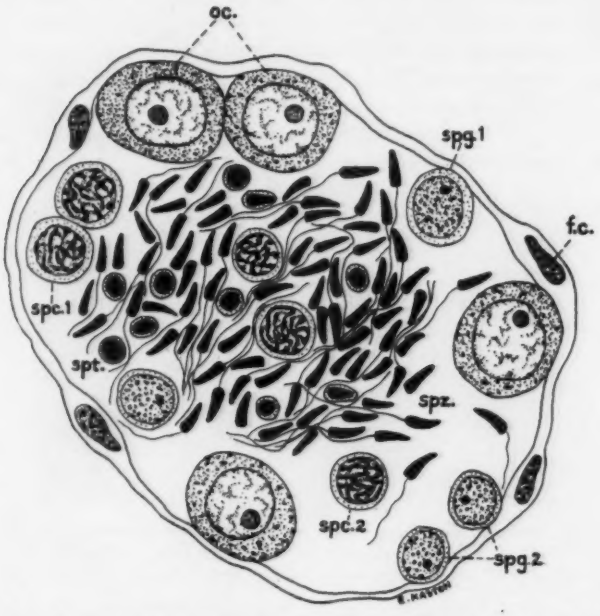


FIG. 6. Bisexual primary gonad of a young individual about 16 weeks of age showing the cells of both sexes but predominantly of male type. *spg.1*, primary spermatogonia; *spg.2*, secondary spermatogonia; *spc.1*, primary spermatocytes; *spc.2*, secondary spermatocytes; *spt.*, spermatids; *spz.*, spermatozoa; *oc.*, large ovocytes showing mitochondrial bodies and yolk nuclei; *f.c.*, follicle cells.

changes of only minor importance occur in the primary gonads. There is apparent neither increase in the number of cells constituting the gonad, nor increase in the size of the follicles. Spermatogenesis is virtually discontinued and cells of the intermediate stages are few in number. The spermatozoa, however, are retained in large numbers in many of the follicles. Unless carefully examined, many of the animals at this stage may be mistaken for true males, but the presence of young ovocytes showing distinct mitochondrial bodies and yolk nuclei indicates the actual

bisexual character of the gonads. In other cases the bisexuality of an individual is more easily noticed because of the large number of young ovocytes present (Fig. 6). A few pycnotic cells are sometimes seen.

Hibernation continues until the middle of April. The lowest temperature of the year is reached in February or the first part of March. After the middle of March the water temperature begins to rise very slowly and reaches 5.0° C. by the middle of April.

Condition in Spring and Summer

By the time the water temperature reaches 7.0–8.0° C., which usually takes place at the end of April, some minor changes in the gonads of young animals begin to occur. They are manifested by resumption of spermatogenesis in some of the individuals. The process, at that time, is very slow, and newly-formed cells, mostly spermatocytes of the first and second order, are few in number. The majority of animals still have gonads in the state resembling that of the hibernation period. As soon as the water temperature advances to a 10° C. mark, usually by the middle of May, more pronounced changes take place in the gonads. Active spermatogenesis is resumed in the follicles of many animals. Numerous gonia, primary and secondary spermatocytes are formed but few spermatids are seen. In many follicles spermatozoa retained since the preceding autumn are present. At this time a slight extension of the follicle is already noted. Phagocytic-nutritive cells begin to surround the follicles in which gametogenic activities are going on. Gonads continue to retain their bisexual nature because many small ovocytes measuring 20–30 microns can always be found in all follicles, even those showing a distinct preponderance of spermatogenic cells. Young ovocytes are, as a rule, found lying in contact with the follicular wall. Their large nuclei make them easily distinguishable from other cells.

Phagocytic-nutritive cells are often found in large numbers along the outer walls of the gonad follicles and a few penetrate into their lumens (Fig. 7). These cells, measuring up to about 12–14 microns and having a nucleus 4–4.5 microns in diameter, occur as a rule near the follicular wall. Their numbers appear to vary with the seasons, the greatest number being found during the active stages of gametogenesis. They are found during all stages of development from indifferent gonad to mature stages. Often some of them project through the follicular walls into the lumen, while others invade it. In clams such cells appear to perform functions of two types. First, their intimate contact with the gonad during the most active stages of gametogenesis indicates that they contribute certain substances necessary for the developing cells. Thus, their nourishing function may be assumed. Second, their pres-

ence inside the gonad follicles immediately after spawning suggests their purely phagocytic rôle of removing partially cytolysed, degenerated and residual cells.

As the season advances and the water temperature gradually increases, reaching about 15.0° C. by the end of June, rapid proliferation of sex cells progresses in a parallel manner. Gonads of young animals examined at that time of the year show that spermatogenesis proceeds very actively and that spermatogenetic cells in all stages of development are filling the follicles, often occupying the entire space of the lumen. It often happens that various follicles of the same animal

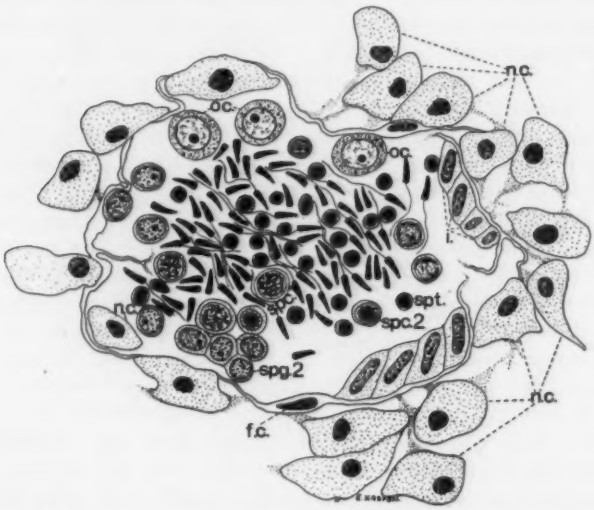


FIG. 7. Gonad of the young clam (1.0 cm.) surrounded by the nutritive phagocytic cells (*n.c.*). Other letters as in Fig. 6. Late April–May.

show quite different stages of development. Frequently one of the follicles possesses a large number of spermatozoa, while another follicle, situated next to it, has most of its cells in early stages of spermatogenesis. Nutritive cells are quite numerous, sometimes entirely surrounding the follicle (Fig. 7). The clams are feeding and growing rapidly, many of them showing an increase in total length of shell of 3 mm. since the end of the hibernation period.

On examining the clams passing through active gametogenesis, one's attention is immediately drawn to the large numbers of nutritive cells surrounding the outer walls of the follicles. In the follicles themselves

great activities are noticed. They are quite distended and filled with spermatogenetic cells. At this time mature spermatozoa are already more numerous than cells of other stages. Spermatocytes in all stages of development and spermatids in spermioteleosis are found. Regardless of such spermatogenetic activities and the distinctly male character of gonads, young ovocytes are always found in small numbers along the wall of the follicles. There is a rapid extension of the branching system of follicles, but this extension is directed chiefly in the posterior direction and not ventrally; consequently very few follicles extend below the level of the stomach.

As has been mentioned before, some of the animals examined during the winter had follicles virtually devoid of all except indifferent cells. Such animals apparently discharged their spermatozoa in the fall. In June and July those animals also exhibit gametogenetic activities and spermatozoa are rapidly formed. It is very interesting to note, however, that in such cases the follicles usually contain more female cells than the follicles of the animals which retained spermatozoa throughout the winter. The variation in the proportion of cells characteristic of the opposite sexes found in clams of the same age strongly indicates that genetic factors and probably the effect of environment influence the production of sex cells.

Spawning

In August the water over the natural clam beds reaches a temperature high enough to induce the spawning of clams. If young clams, which by this time have reached the size of 2.0–3.0 cm. are examined, their gonads reveal the fact that the spawning process is not a phenomenon of short duration but lasts for some time. Different follicles of the gonad of the same animal contain sex cells in various quantities. Some follicles have already discharged their contents while in other follicles spermatogenesis is in progress and the lumen is still filled with mature spermatozoa. Apparently, several days, or perhaps weeks, are required for young clams to complete their spawning. Furthermore, by examining a sample consisting of many animals, the conclusion can be reached that there is considerable difference in the spawning behavior of individuals because some of the animals collected at the same time and from the same place have their sex products completely discharged, while others still retain their spawn in various quantities. This indicates that the entire population of a certain bed does not begin spawning at exactly the same time, and that the spawning season of clams extends for a considerable period of time.

In many follicles, which during the spawning period are partly or

fully freed of spermatogenic cells, young ovocytes, many of them in the spireme stage, are growing along the walls. In some instances groups of ovocytes occupy considerable portions of follicles. A few nutritive cells may be observed in the vicinity of follicles whose contents are already discharged. Branching gonad tubules begin at this time to extend ventrally, occupying the space between the body walls and the digestive gland.

During the months of August and September the animals grow very rapidly, reaching the size of 2.5 to 3.2 cm. Many animals have grown as much as 2.0 cm. since the end of the hibernation period. Simultaneously with the growth of the animals, ramification of gonad follicles proceeds. The follicles spread in all directions and envelop the stomach and intestines as well as the spaces in connective tissue between the body wall and the digestive gland.

Transformation to Definitive Males and Females

In September, after the spermatozoa have been discharged, two types of individuals become distinguishable as definitive males and females. In the males a second or third period of spermatogenesis begins in the autumn and continues at a reduced rate throughout the winter. Mature spermatozoa are retained in the follicles all this time. Many young ovocytes and numerous indifferent cells are also present. In the spring, with the increase of water temperature, renewed rapid branching of follicles takes place and the gonads then begin to acquire the typical male character of the adult. Spawning follows later in the summer.

In animals destined to become females radical changes take place in the gonads upon the completion of the initial male phase. After the discharge of spermatozoa the follicles are left in a distended state. Their lumens are large and empty (Fig. 8) with the exception of a few pycnotic bodies consisting of cytolysed spermatogenic cells. An irregular layer of indifferent cells, ovogonia and primitive ovocytes measuring up to 20–25 microns, and a few spermatogonia remain for a time along the walls of the follicles. Nutritive cells lie in close contact with the gonad walls and occasionally can be found inside the lumens of the follicles. No significant changes occur in the gonads of future females during the hibernation period, for during all this time the follicles remain distended, round or oval in outline, with virtually empty lumens. There is little indication of ovogenetic activities. In the middle of May, regardless of the fact that the water temperature is 9.7° C. (i.e., about four degrees above hibernation mark), and that the animals already had been feeding for approximately one month, their gonads remain in a semidormant

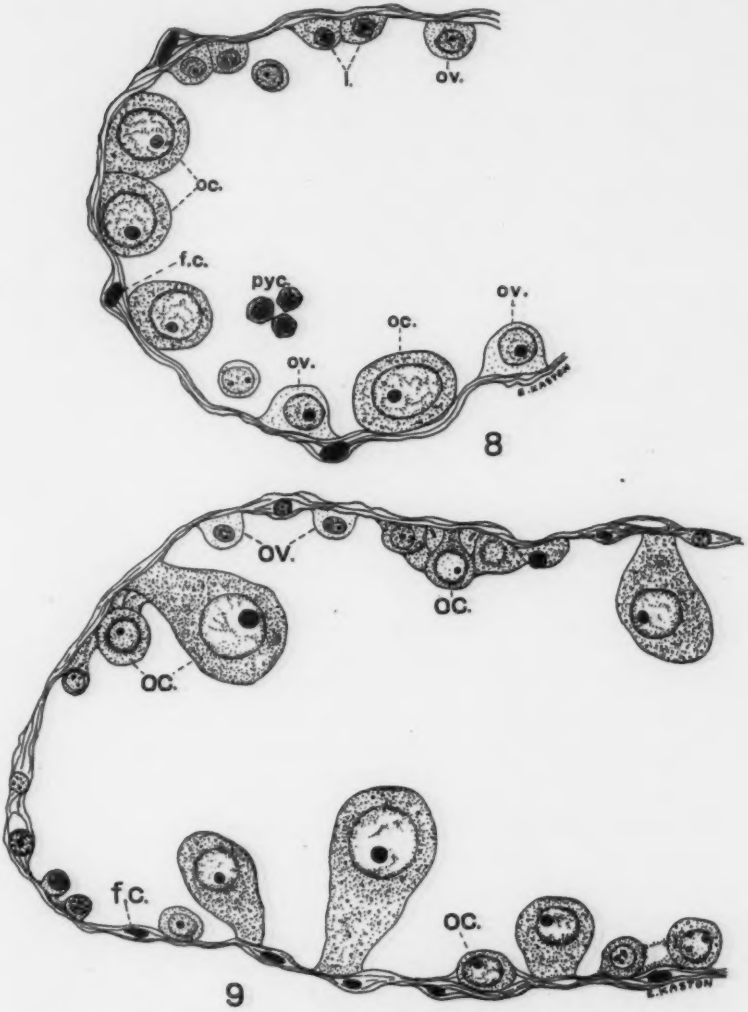


FIG. 8. The gonad of the animal destined to become a female after the completion of the initial male phase. *I*, indifferent cells; *ov.*, ovogonia; *oc.*, young ovocytes; *pyc.*, pycnotic cells; *f.c.*, follicle cells.

FIG. 9. Early ovogenetic activities in the young female clam producing its first crop of eggs; *ov.*, ovogonia; *oc.*, ovocytes in various stages of development; *f.c.*, follicle cells. Age 18–19 months. Middle of May.

state. Only in rare instances are synaptic activities in young ovocytes noticed. In some follicles, however, pycnosis and phagocytosis of spermatogenetic cells left over from the last autumn proceed very rapidly.

In clams producing their first crop of ova, slow ovogenetic activities become apparent in the middle of May and active ovogenesis begins in June when the water temperature approaches 15° C. Within a few days after such a temperature has been reached the appearance of the gonads undergoes a marked change. Young ova in various stages of development, some of them 42–45 microns in size, grow from the walls of the follicles into the lumen (Fig. 9). The growth and proliferation of ova are very rapid and the animals formerly functioning as males have now reached the stage of functional females. Not all the follicles begin to produce ova at the same time. Frequently, in the same animal some of the follicles contain large, rapidly growing ova, while a few others are still in an apparently dormant state showing many indifferent cells along their walls, ovogonia and very minute ovocytes.

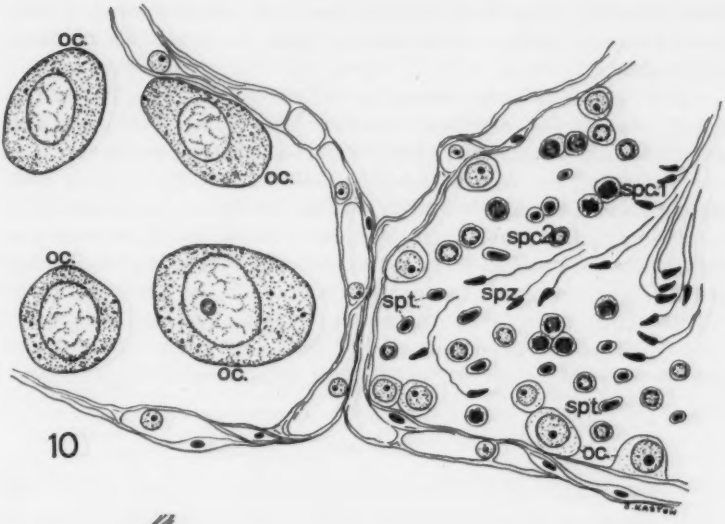
The first crop of eggs produced by the young female is usually small and in this respect they can always be distinguished from fully adult individuals, because in the latter case the follicles contain many more ova. After spawning, the gonads of both sexes may contain some cells characteristic of the opposite sex, thus retaining their bisexual character. It is especially noticeable in the case of males, where comparatively large ovocytes are easily distinguished from male cells.

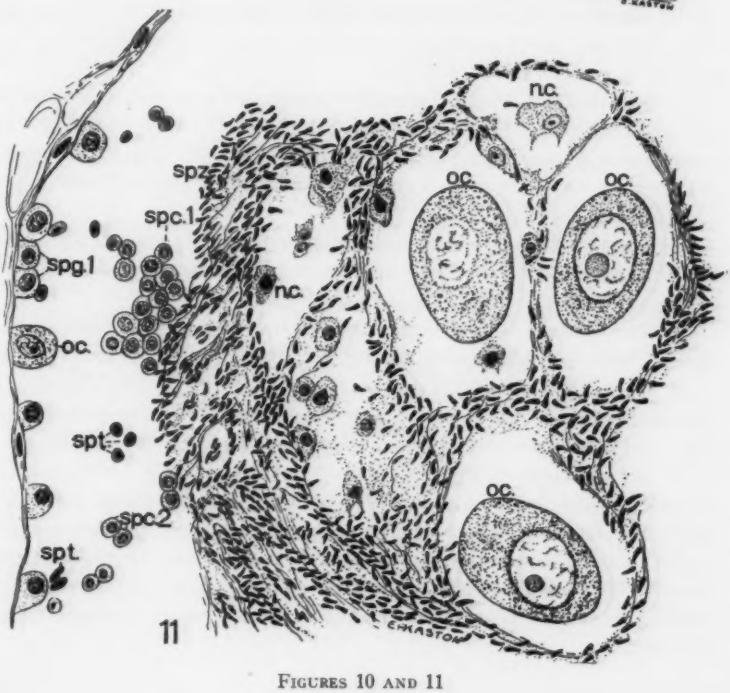
Functional Hermaphroditism

Pelseneer (1926) has shown that hermaphroditism is quite common among mollusks. Among Pelecypoda numerous cases of partial and true hermaphroditism have been reported. As has been stated in the chapter on the development of the primary gonads of *V. mercenaria*, many grades of bisexuality occur in that animal during the first two years of its life. The examination of the gonads of adult males also reveals, in almost every instance, the presence of small ovocytes somewhere along the walls of the follicles. In all such cases, since the cells of both sexes are present in the gonads of the same individual, the term "partial hermaphroditism" may be applied. However, under functional or true

Fig. 10. Functional hermaphroditism in *Venus*. Portions of two adjacent follicles of the same individual containing cells of opposite sexes. *spc.1*, primary spermatocytes; *spc.2*, secondary spermatocytes; *spt.*, spermatids; *spz.*, spermatozoa; *oc.*, large ovocytes showing mitochondrial bodies and yolk nuclei.

Fig. 11. Portion of hermaphroditic gonad showing the ripe ova surrounded by spermatogenetic cells. *spg.1*, primary spermatogonia; *spc.1*, primary spermatocytes; *spc.2*, secondary spermatocytes; *spt.*, spermatids; *spz.*, spermatozoa; *oc.*, large ovocytes showing mitochondrial bodies and yolk nuclei.





FIGURES 10 AND 11

hermaphrodites, only those animals should be included whose gonads possess masses of cells of both sexes, in which case self-fertilization may be possible.

In *V. mercenaria* the adults are, with few exceptions, of separate sexes. Among 650 mature clams studied by means of serial section, only 3 cases of functional or true hermaphroditism were observed. In one case fully ripe ova were found in some of the follicles, while spermatozoa occupied an adjacent but separate portion of the gonads (Fig. 10). In two other cases a few ripe ova were occupying the lumen of the follicle in which spermatogenetic activities were also almost completed. Numerous ripe spermatozoa surrounded the ova lying in the follicles (Fig. 11). In all of these instances the ova and spermatozoa may be discharged simultaneously into the mantle cavity of the animal and then into the water where abundant opportunity for self-fertilization offers itself.

Summary

1. Examination of the developing gonads of young clams from Long Island Sound, at two-week intervals during the first year of life, shows that a primary bisexual gonad is formed in each individual when the animal reaches the size of 4–6 mm.

2. The primary gonads form from associations of germinal cells in the connective tissue, not immediately beneath the body walls but at some distance from them, at the level of the heart or slightly below it. A lumen soon appears in each gonad follicle and the germinal cells begin to proliferate rapidly.

3. The primary gonad contains the antecedent cells of both sexes but the protandric nature of the primary gonad becomes manifested by the rapid proliferation of the spermatogenic cells.

4. This species is not strictly protandric because few individuals develop into females without passing through a functional male phase.

5. Functional spermatozoa have been observed in the gonads of young clams, collected in October and November when only 5–7 mm. long. In some cases discharge of spermatozoa occurs at that time.

6. In September of the second year, after the animals have spawned as males, two types of individuals become distinguishable as definitive males and females. In the males a second period of spermatogenesis begins in the autumn and continues at a reduced rate throughout the winter. The bisexual character of the definite sexual gland is usually retained to at least some extent because of the presence of many small ovocytes.

7. In animals destined to become females, after the completion of the initial male phase, the gonads may remain virtually empty but in a distended state throughout the winter. There is little indication of ovogenic activities until the following spring when the water temperature approaches 15° C. From then on the proliferation and growth of ova proceeds very rapidly until the animal reaches the functional female phase.

8. In *V. mercenaria* the adults are, with few exceptions, of separate sexes. Among six hundred and fifty mature clams studied by means of serial sections only three cases of true hermaphroditism were observed.

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SEASONAL GONADAL CHANGES OF ADULT CLAMS, VENUS MERCENARIA (L.) [1]

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INTRODUCTION

The literature on Pelecypoda mollusks contains but few accounts of systematic studies of the changes occurring in the gonads of an animal throughout the entire year. This phase of investigation of sexual phenomena in mollusks, though neglected so far, is, however, of considerable importance and interest because, if properly carried out, it could assist greatly in the understanding of the phenomena of change of sex in some forms or add to the knowledge of the alternation of sexual phases in others. Amemiya (1929) reported a very interesting case of sex change in the Japanese oyster, *O. gigas*, which was previously considered dioecious. His method consisted of making a hole in the shell of an oyster and removing through it a small piece of gonad for determination of sex. Following this the oysters were placed in cages and returned to the water for a period of one year, at the end of which they were re-examined. Meanwhile, no samples were taken for histological studies to determine what cellular changes precede the reversal of sex. Nelson (1928) gave a more or less detailed description of changes occurring in the gonads of the adult *O. virginica* from April until June, but did not continue his studies through the post-spawning time and season of hibernation. In other studies dealing with sexual phenomena of the genus *Ostrea*, a systematic description of changes occurring in the gonads of adult animals throughout the year is also lacking.

This paper deals with the changes occurring in the gonads of adult clams (*Venus mercenaria*) at different seasons of the annual cycle. The animals were studied for three successive years. Samples for this study were collected at bi-weekly intervals from the clam beds located near Charles Island, Long Island Sound. The depth of the water over the beds was 8–12 feet at mean low water.

Since the calendar's new year, occurring in the middle of winter, coincides with the prolonged period of the animal's inactivity, it is thought convenient in this study to consider the beginning of a new year from the ecological point of view, i.e., after the completion of

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spawning activities. It so happens that the spawning of *V. mercenaria* in Long Island Sound occurs at the time when the water temperature reaches its highest mark of the year. Ecologically this also represents the terminal point of the annual cycle. Therefore, immediate post-spawning time will be considered as the beginning of the ecological year, and the description of the changes occurring in the gonads of *V. mercenaria* will begin at that date. Sexes will be considered separately, the changes occurring in male gonads being described first.

The writer desires to express his appreciation to Professor W. R. Coe for his advice and criticism throughout the course of this work.

Males

The spawning of *V. mercenaria* is usually completed at the end of August or in the early part of September. The gonads of males examined during that period show large distended follicles containing but few sex cells. The center of the lumen is empty and the few spermatozoa that are still left remain near the walls of the follicles undergoing cytolysis. Spermatids and spermatocytes of both orders are virtually absent from all the follicles, but large primary gonia, a few secondary spermatogonia, indifferent cells and, occasionally, young ovocytes are commonly found lying in contact with the follicular walls. In many instances large, deeply-stained pycnotic cells, apparently the product of abnormal spermatogenetic activities, may be seen.

Male clams examined late in September and early in October reveal that the post-spawning period of recuperation has already passed, and the gonads of the animals are rapidly producing sexual cells for the next year. The gonad follicles at that time are occupied with spermatogenetic cells in various stages of development. Along the walls of the follicles numerous spermatogonia grow rapidly, often forming two or three rows of cells. Further in the lumen, occupying nearly all the available space, spermatocytes of both orders and spermatids in early stages of spermioteleosis can be seen. Fine protoplasmic strands connect the cells with the follicular walls. Undoubtedly the nutritive materials are delivered to the developing cells through these strands. In the majority of cases no ripe spermatozoa have yet been formed in the follicles. It is very characteristic that at this time, as well as at all other times of the active gametogenetic activities, the follicles are surrounded by a great number of nutritive-phagocytic cells. The water temperature over the clam bed at the time of collection of the samples was about 19.0° C.

By the middle of October the bottom water temperature of Long Island Sound decreases to about 16.0° C. Examination of the gonads

at that time shows that spermatogenesis is continuing and that spermatozoa have already been formed in many of the follicles. As usual, numerous individual variations can be observed in the numbers of spermatozoa found in different follicles. Some of the follicles have the largest part of the lumen already occupied by apparently mature spermatozoa, while in others few cells in such an advanced stage can be seen. In general, the cells of the early stages of spermatogenesis still predominate. By the end of October (water temperature 12–13° C.) spermatozoa can be found in virtually all the follicles.

Regardless of the fact that during November the temperature of the water decreases rapidly, reaching about 8.0° C. at the end of the month, the spermatogenic activities of the clam proceed without interruption. By this time spermatozoa are by far the most numerous cells in the follicles. Spermatids in all stages of spermioteleosis also are present in large numbers, while spermatocytes and spermatogonia are much less numerous than in the animals examined in October and the early part of November. Many spermatocytes are seen in the synaptic stage or in the process of division. Practically the same condition exists in December. In all cases a large number of pycnotic cells are found scattered among the healthy ones and the phagocytic cells are actively engaged in clearing them from the follicles.

The middle part of December may be considered as the threshold of the winter hibernating period of *V. mercenaria* of Long Island Sound (Loosanoff, 1936*a*). The water temperature by that time decreases to a critical point of about 5.0° C. and the animal, confronted by an unfavorable environment, undergoes important physiological modifications which induce hibernation. But if the gonad sections prepared from animals collected during the early part of the hibernation period are compared with those collected one or two months previous to its onset, little difference can be noticed in their character. Throughout January and February spermatogenetic activities are continued at a reduced rate.

Animals collected in February possess large sex glands, enveloping the stomach, liver, intestine, and penetrating into the foot. The follicles are fully extended and filled with spermatogenetic cells of which spermatozoa are the most numerous. Excluding a brief post-spawning period, the spermaries of adult males of *V. mercenaria* contain morphologically mature spermatozoa at all seasons of the year. Spermatozoa, if removed from the spermaries and placed in a dish of sea water, after 2–3 minutes of quiescence begin to swim actively in their typical spiral way. This simple experiment was tried at bi-weekly intervals throughout the year and always, except in the short post-spawning period, gave

positive results (Loosanoff, 1936*b*). The question of physiological ripeness of spermatozoa found in clams at all other times, except prespawning and spawning periods, still remains open because no eggs capable of fertilization could be obtained for such an experiment in late fall, winter and spring.

The month of March may be regarded as the time of the year when the spermatogenetic activities of the clam are the least conspicuous. The spermaries of animals collected during that month contain mainly spermatogonia and spermatozoa, while the cells of the intermediate stages are present only in small numbers. In the middle of April the water temperature passes above the critical hibernation point for clams. Nevertheless, as far as production of gametes is concerned, the animals do not respond noticeably to the favorable change in environment. The gonads remain quiescent, showing little spermatogenetic activity until the middle of May, when the water temperature begins to fluctuate between 10.0° and 13.5° C. As soon as the temperature reaches 15.0° C., which in Long Island Sound takes place early in June, vigorous spermatogenesis is again initiated and production of spermatozoa proceeds at an extremely rapid rate. The presence of a large number of spermatids in the follicles is very characteristic of this part of the year. Simultaneously with increase in water temperature further increase in activities of spermaries is noted. In June, July and during the first part of August many animals are found with ripe gonads. In several cases it was possible to induce such animals to spawn by a gradual increase of the water temperature. Among animals living in very shallow water or on tidal flats, individuals with partly discharged gonads may be found early in July, but the largest part of the clam population spawns in August when the curve of water temperature reaches its culmination point.

Females

During the immediate post-spawning period, after the gametes have been discharged, the sexes of *V. mercenaria* can still be distinguished easily. A description of the post-spawning condition observed in the clam spermaries has already been given. The ovaries in the post-spawning stage are characterized by the presence of a few unspawned, but apparently ripe, ova and by a large number of ovogonia and very young ovocytes. The presence and normal appearance of these ovocytes during the stage when sex reversal may occur in mollusks, points toward the conclusion that in *V. mercenaria* the change of sex from female to male seldom takes place.

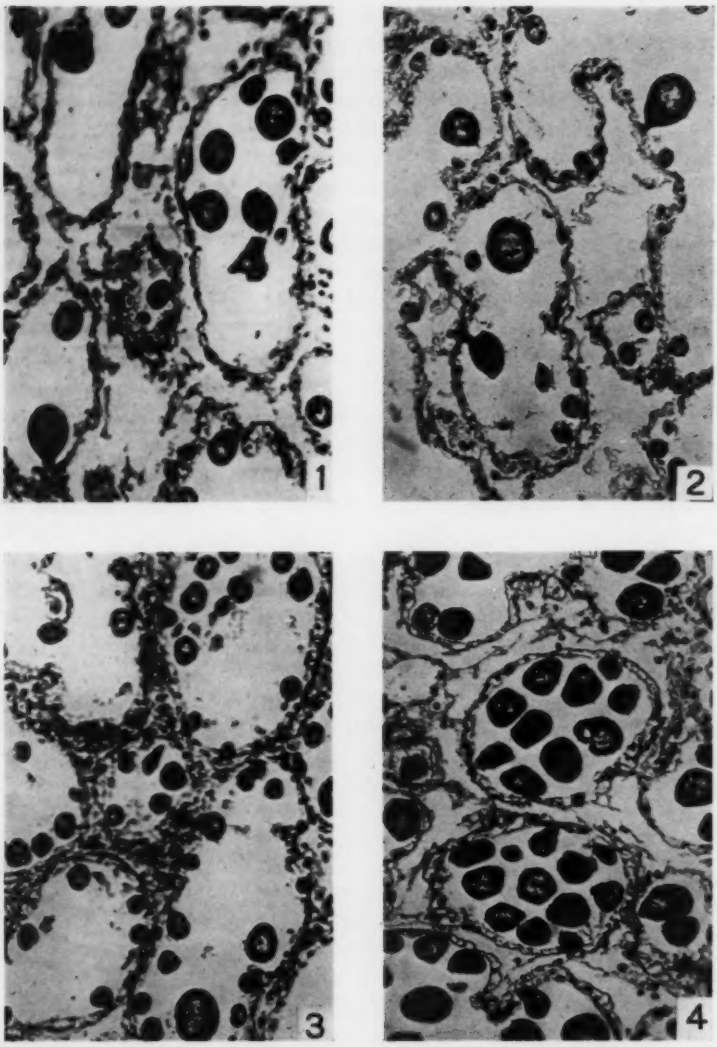


Fig. 1. Female gonad immediately after partial spawning. Follicles with unspawned ova are in a semi-contracted state. Early August. × 300.

Fig. 2. Female gonad in September. The ovarian follicles are still in a contracted state. Many young ovocytes are growing. A few unspawned ova still remain. × 300.

Fig. 3. Female gonad in October. × 300.

Fig. 4. Female gonad in November, showing the rapid growth of young ovocytes. × 300.

The number of large unspawned ova found in the ovaries of recently spawned clams varies greatly with individuals. In some cases almost the entire crop of ripe ova is discharged by the time spawning activities end, while in the majority of cases, a comparatively large number is retained. The difference between the follicles of the same animal, as to the number of unspawned ova left, is often very marked. Almost immediately after spawning the ovarian follicles begin to contract. This contraction does not proceed very far, however, and they are left in a semicontracted state with unspawned ova still retained in the lumen (Fig. 1). An almost continuous layer of ovogonia, indifferent cells, and young ovocytes in various stages of development and growth is often distinguished along the inner walls of the ovarian follicles.

The spawning of an individual clam is not completed in one attempt but is extended for a certain period of time, depending upon the individual and ecological peculiarities. Female clams with partly discharged sexual products were found on our experimental bed throughout August and September. Their ovaries contained, in various proportions, the cells of old and new crops. Towards the end of September only a few ripe ova were found. Apparently a delayed but nevertheless normal discharge of eggs takes place this late in the season.

The discharge of the ripe eggs evidently removes the factors inhibiting the production and growth of a new crop of ovocytes. As soon as the ovaries are freed of the bulk of their ripe eggs, ovogenetic activities are commenced. At first a few young ovocytes are seen growing and protruding towards the ovarian cavity (Fig. 2). The new ovocytes at this time vary markedly in size. The average size is approximately 17–20 microns but some of the youngest ones measure only 8–9 microns, and the largest, which are less numerous than others, reach the size of 35–40 microns. In almost every case studied the ovarian follicles were found to contain a few very large ovocytes measuring 55–60 microns. Such ovocytes, many of which were still connected by their egg stalks to the ovarian walls, represented the cells of the old crop.

Early in October a considerable increase in the number of new ovocytes is observed. Many of these ovocytes are still of a rather small size as compared with fully grown eggs, a few of which are left in the ovaries (Fig. 3). At this time the average size of a new crop of ovocytes is about twenty-five microns. It should be remembered, however, that individual differences are great. It is often found that in a sample composed of a large number of animals a few individuals may have their gonads in either a greatly retarded or an advanced stage, as compared with the conditions observed in others of the same samples. This

is sometimes very confusing and unless the sample studied is composed of a large number of animals, erroneous conclusions may be reached.

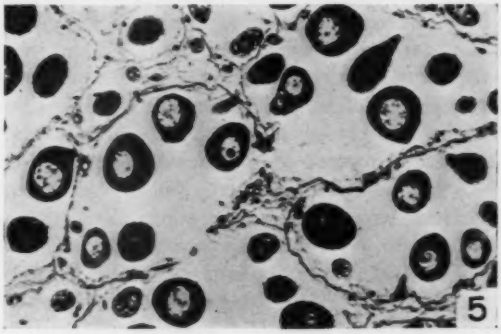
During the latter part of October and in November the growth of young ovocytes is very rapid. Some cells of the new crop have already reached the size of a mature egg but most of the cells are between 33 and 55 microns in size. There are still many minute ovocytes making their appearance but their number is considerably smaller than it was in the early part of October (Fig. 4). Simultaneously with the growth of the ovocytes, the follicles expand and often proliferation and formation of new follicles in the surrounding connective tissue can be observed. The young sex cells of such follicles are much smaller in size than those of the old ones. In November the discharge and absorption of all unspent eggs are quite completed. In only exceptional cases can such cells be found at that time.

Orton (1933) found that in most cases the unspent eggs of *O. edulis* are extruded from the gonad within a few days after the principal spawning, while a small percentage of individuals may retain the remaining ova for as long as two months and then discharge them in the usual way. In the case of *V. mercenaria* the fate of some unspawned ova is probably similar to the fate of those in *O. edulis*. Studies of the gonads of *V. mercenaria* in post-spawning stages reveal that the number of undischarged eggs in the ovaries gradually diminishes as the season progresses. Some of the unspent ova found in the ovaries of clams during September–November are normal in appearance, having no indication of any decomposing processes going on. The fact that few if any phagocytes are observed in lumens of the ovarian follicles during the post-spawning period also adds weight to the conclusion that some of the ova unspent during the spawning season are extruded later in the normal manner. Such extrusion is completed in November.

Regardless of the fact that the temperature of the water over the clam bed decreases very rapidly during November, and in December falls below the point at which hibernation of clams begins, the growth and development of gametes proceeds normally. Ovaries of clams collected late in December already contain large numbers of ova which have a virtually mature appearance (Fig. 5). During that part of the season ovarian follicles contain relatively but very few of the minute ovocytes which were so characteristic for the gonads of early fall. It is quite clear that the young ovocytes which appeared in the follicles after completion of spawning have grown by now to a large size, and that since the appearance of the first and numerous group of ovocytes in September and October but few new cells appeared later on.

As may be seen from Fig. 5, in December samples large ovocytes

protrude into the lumen occupying much of the available space in the follicles. A few pycnotic cells, some of them ovocytes in very early stages of growth, may often be noticed. It is very strange that during this time as well as the entire post-spawning period, when the ovogenetic activities of clams are at their height, very few nutritive cells are present near the follicles containing growing ovocytes. In the development of



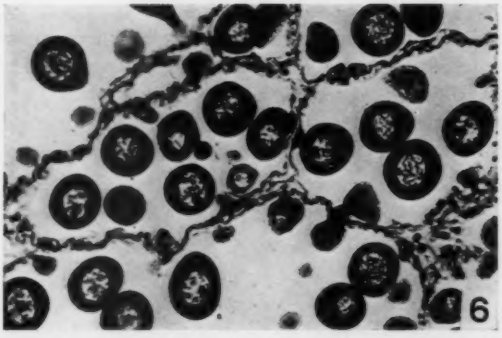


FIG. 5. Female gonad in December, containing large number of ova of mature appearance. × 300.
FIG. 6. Female gonad in May, containing ripe ova. × 300.

male gametes the rôle of such cells is of considerable importance since it is apparent that they participate in providing nutrition for the developing sex cells. In female clams ovogonia and ovocytes continue their growth mainly by extracting nourishment from the mesenchyme and blood vessels between the follicles. The nutritive cells, so important in male gonads, are much less numerous and apparently of lesser significance in the metabolic cycle of the ovary.

During the winter months, January–March, few changes occur in clam ovaries. The slow growth of young ovocytes continues but at a much reduced rate. There are few if any new young ovocytes beginning to develop from ovogonia during this time of the year. Large ovocytes, constituting the majority of the cells of the ovaries, measure up to 66–68 microns, i.e., equaling the size of the mature cells. Occasionally pycnotic cells may be noticed in one of the follicles, but as a rule there is little evidence of pycnosis. During April and early May the growth of the undersized ovocytes continues. By the middle of May the ovaries acquire a fully ripe appearance with approximately 90 per cent of all the cells reaching their limit in size which varies between 66 and 70 microns if the ovocyte is of normal oval shape.

During the second part of May some of the eggs of the clam are mature not only morphologically (Fig. 6) but also physiologically. On May 25, 1934, four large female clams were taken from the bed and brought to the laboratory where they were placed in an aquarium. The temperature of the water over the beds was 14.0° C. and that of the aquarium water 26.0° C. After two hours of exposure to such a temperature two females began spawning. Discharged eggs were fertilized by the addition of sperm suspension and they began to develop in the normal way. Thus, during the end of May, June and July, sexually mature and ripe clams merely wait for the water temperature to reach the point at which the proper stimuli inducing spawning reaction are produced. The general spawning of the clam population of the Charles Island beds occurs most frequently either late in July or early in August, and continues into September.

Discussion

Studies of the changes occurring in the spermaries of adult *V. mercenaria* reveal that the post-spawning period, when the gonads are devoid of all but undifferentiated cells, is of very brief duration. This is probably the only period when the change of sex from male to female can take place. Production of a new crop of spermatozoa begins very soon after the completion of spawning. The main portion of spermatozoa for the next year's crop is produced during the autumn. Mature spermatozoa in small numbers may be found in the spermaries of a few clams as early as the end of September. In November the spermatozoa are already the most numerous cells in the gonad follicles, which at this time acquire an appearance of ripeness. Spermatogenesis continues at a reduced rate throughout the winter but is practically at a standstill during the early spring. The second manifestation of very rapid

spermatogenetic activities takes place in June, when the water temperature reaches 15.0° C. The discharge of spermatozoa follows when the critical spawning temperature of 23–25° C. is reached.

Summarizing the observations of the processes occurring in the ovaries of *V. mercenaria,* it may be stated that the production of a new crop of eggs begins immediately after spawning. The chief growing period of the young ovocytes is in the autumn. In December and January the gonads of many female clams already present a ripe appearance. During the winter and spring only a few new ovocytes begin their development; cells produced in the autumn constitute the greatest portion of the next year's crop. Some ova, undischarged during the spawning season proper, may remain in the ovaries for a considerable length of time, and are finally discharged in the normal way. Many of the ova ripen approximately two and a half months before the water of the clam beds reaches the critical spawning temperature.

Compared with the sexual cycles of other pelecypods, it is rather unusual that in *V. mercenaria* the development of gametes occurs during the season when the water temperature rapidly decreases and the ecological conditions for gonad development appear to be less favorable than in the spring and early summer. According to Coe (1932), low temperature during the winter may delay the completion of the sexual phase in *O. lurida* for several months. A phase may be inaugurated in October and completed in April, while in the summer the same phase might be completed in a few weeks. The same author states that in the autumn ovulation in *O. lurida* is often inhibited. Judging by this it is probable that after the completion of the last phase of the year the animal does not display very active gametogenesis in the following autumn and winter, and that the main production of the gametes of the next sexual phase takes place in the spring when the water temperature begins to increase. In *O. gigas* (Amemiya, 1928) the production of gametes occurs chiefly in the spring. According to observations of the writer, adult individuals of *O. virginica* of Long Island Sound enter into the resting stage soon after completion of spawning and remain sexually inactive until the next spring. In *O. commercialis* (Roughley, 1933) the gonads are found in the resting stage throughout the winter and gametogenesis commences in the spring. Probably the same condition will be found in many other mollusks. In *V. mercenaria,* on the other hand, the most active gametogenesis and the production of the year's crop of sex cells occurs in the autumn and early winter. Since ovogenesis continues throughout fall and winter there is no actual period of recuperation which is commonly observed in other allied forms

living in approximately the same geographical areas and subjected to similar ecological conditions. Retention of morphologically ripe sperm and ova throughout the greatest part of the year is another interesting feature of the sexual behavior of *V. mercenaria.*

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THE RATE OF WATER PROPULSION BY THE CALIFORNIA MUSSEL

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Numerous investigators have approached by means of various experimental techniques the problem of the volume of water which may pass in a given time through the mantle cavity of lamellibranch mollusks. The establishment of an approximate, or even a minimum figure for the volume of water pumped per day by any of the many marine plankton-feeding animals would be of interest to physiologists who may be concerned with nutritional, respiratory, or excretory activities of the animal itself, to planktologists whose problems deal with the numerous factors which influence the numbers and distribution of microscopic plants and animals that may be consumed by the filtering animals, and to oceanographers who seek information regarding biological factors concerned in modifications of the physical and chemical character of both water and bottom in various regions of the sea. In many instances, those who have contributed to our knowledge of the water-filtering and feeding activities of lamellibranchs have been primarily interested in the sanitary and other technical aspects of cultivating oysters and mussels for human consumption. Thus Viallanes (1892) presented approximate figures for the relative rates of filtration of water by French oysters, Portuguese oysters, and mussels. He placed the animals in separate crystallizing dishes upon the bottom of a tank supplied with flowing sea water, keeping initially empty dishes of the same size and design alongside of the experimental ones, in the same tank. At the end of a specified time interval the material which had been filtered from the water by the animals, and either swallowed and later voided as fecal material, or else rejected as pseudofeces directly from gill and mantle surfaces, was collected, dried, and weighed. The weight of filtered material was obtained by subtracting that of the detritus which had settled out of the water by gravity into the control dishes. Viallanes performed similar experiments more critically by dispersing a known quantity of dry clay in a given volume of water, drying and weighing the quantities of clay deposited by the different bivalves after 24 hours. From his first experiment, wherein

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the suspended material naturally present in the sea water was the only available particulate matter, he calculated that his animals (of age = 18 months) filtered water at the following relative rates: French oyster, 1.0; Portuguese oyster, 5.5; mussel, 3.0. The clay experiments, however, showed very different results, which were left undiscussed. Table I shows briefly his results.

Viallanes found that the mucus which was secreted by the mollusks and in which the precipitated clay was incorporated, weighed only 4 per cent of the weight of the whole mass.

Ranson (1926) cites the work of Viallanes and reports briefly his studies of the mechanism of filtration. Both investigators emphasize the importance of the filtering function of lamellibranch plankton- and detritus-feeders to ostreiculturists and to oceanographic science.

TABLE I

Filtration and deposition of suspended clay by Viallanes' lamellibranchs.

Animal	Initial quantity of dry clay	Weight of clay deposited in 24 hours	Minimum volume of water filtered per hr. (our calculation)
	gram/liter	*grams*	*liters*
Mussel (18 months)	0.0546	1.768	1.35
Portuguese oyster (18 months)	0.0546	1.075	0.82
French oyster (18 months)	0.0546	0.199	0.015

Criticisms of Viallanes' experiments which must be borne in mind are: (1) in his first experiments with naturally-occurring particulate matter, the portion of the material which had been actually swallowed, the undigested residue of which was finally voided as fecal material, must have undergone changes of various kinds and degrees depending upon the relative rates and processes of digestion in the different species, and have subsequently vitiated the interpretations that were based merely upon relative weights; (2) in both of the experiments, the quantities of detritus or clay which had been removed from the water but still remained in the animals' alimentary tracts were not weighed or considered. Since in each case such small total weights were being measured, this item might have constituted a considerable, though by no means predictable or constant fraction of the total figure.

Galtsoff (1926, 1928*a*, 1928*b*) measured the rate of flow of water through the gill chamber of the American oyster, and calculated the work done by the propelling cilia. He used two methods, one a direct one in which the water issuing from the exhalant chamber was collected in a measuring vessel, the other in which the rate of advancement of a

stream of water flowing from the exhalant chamber through a glass tube was measured by stop-watch. Both methods involved the insertion of a small glass rod between the opened valves to prevent their closing, and placing into the gill cavity a rubber tube, to carry off the discharged water, packing other open spaces around the tube with cotton. Galtsoff (1928*b*) records a maximum figure of about 3.9 liters per hour for the water intake of a single healthy adult oyster three to four inches in length at a temperature of 25° C.; the temperature at which the highest rate of flow occurred was found to be between 25° and 30° C. He points out, and shows by collected data the fact that considerable variations exist in the rate of flow produced by individual oysters. His average temperature-flow rate curve, taken from data collected in the study of many individuals, shows, at temperatures close to 30° C., a maximal rate of about 2.4 liters per hour while at 20° C. the average rate lies close to 2 liters per hour. At temperatures between 24 and 27.9° C., filtration took place at the rate of from 2.5 to 2.9 liters per hour. He points out that the filtering action of the oyster is dependent upon two mutually independent functions, namely the beating of the cilia and the opening and closing of the valves of the shell, the process occurring only when the valves are apart and the cilia beating. His studies showed that the oyster keeps its shell open for an average time of about seventeen hours out of twenty-four. (June to October.)

Numerous attempts have been made to estimate the rate of flow of water through the gill chambers of lamellibranchs on the basis of plankton counts in the water itself, and in the animals' stomachs. The results are, however, not satisfactory because, as Galtsoff (1928*b*) points out, there are daily and seasonal variations in plankton numbers in the water; not all of the diatoms filtered out by the gills are ingested but may be rejected instead. Such experimental work should, however, if carefully controlled, afford minimum figures. It would seem for various reasons probable that most methods used to date might be expected to yield results that are lower than the true figure for the volume of water filtered per day.

Various investigators (see also Galtsoff, 1928*b*) have obtained widely different results for the rate of water-pumping by plankton feeders. Collateral data regarding temperature are, however, not always available. Briefly, the general conclusions may be recorded as shown in Table II.

The inconsistencies which appear from an examination of these figures serve to emphasize the wide variations according to the method used, and the unreliability of the plankton count method. Galtsoff's studies convinced him that as many as 18.9 per cent of phytoplankton

such as the diatoms *Chætoceros* and *Rhizosolenia,* and the dinoflagellates *Peridinium oceanicum* and *Ceratium* might escape being caught while passing through the gills, and that from 50 to 89 per cent of bacteria present in the water passed the gills of his oysters. Work at this Institution, however, shows that the mussel is quite successful in removing bacteria from water. Thus ZoBell and Landon (1937) demonstrated that the mussel removed about 99.9 per cent of added bacteria from sea water. (See also Fox et al., 1936.)

Without doubt, Galtsoff's direct methods are the most accurate for

TABLE II

Filtration of water by plankton-feeders.

Investigator	Animal	Method	Average rate of filtration (liters per hour per animal)
Grave (1905)...	Oyster	Plankton counts in water and stomachs	0.167
Moore (1913)..	Oyster	Plankton counts in water and stomachs	ca. 1.25
Allen (1914)...	Freshwater mussel	Rubber tube packed into exhalant chamber	1.4
Wells (1916)...	Oyster	Plankton counts in water and stomachs	7.5
Nelson (1921)..	Oyster	Rubber apron dividing inhalant and exhalant chambers	5.7 (but see text below)
Galtsoff (1926, 1928*a*, *b*)....	Oyster	Rubber tube packed into exhalant chamber	2.5–2.9 at 24–26.9° C.
Dodgson (1928)	Mussel (*Mytilus edulis*)	Clearing of suspensions	2.0 (minimum, at 17° C.)
Parker (1914)..	Sponge (*Spinosella*)	Glass tube tied into oscu-lum	3.2
Damas (1935)..	Cardium	Clearing of mud suspensions	0.1

the measurement of the rate of flow of water through the mantle cavity under the conditions imposed upon the oyster. Needless to say, a question arises in one's mind regarding the possible influences that blocking the valves apart with a glass rod, inserting a rubber tube into the excurrent chamber, and packing the openings in the mantle surrounding the inserted tube with wads of cotton might exert upon the normal feeding and filtering behavior of the animal.

Nelson (1935) refers to earlier efforts to measure the water filtered by an oyster, and to the fact that very diverse results were obtained. He writes, " The introduction of a tube into the cloacal chamber inter-

feres with normal operation of the branchial hearts described by Hopkins and may disturb the visceral ganglion." He adds, "Also, in *Ostrea virginica* much of the water from the right demibranch leaves by an asymmetric chamber on the right side and separate from the cloacal chamber." Using a modification of the rubber apron of Moore (1908), he claims to have measured all the water passed by an oyster, without interfering with its normal activities. He reports the amazing value of 26 liters of water per hour, passed by an oyster 11.5 × 8.9 cm. in size, at the optimum temperature of 30° C. This is about tenfold the average values found by Galtsoff for the oyster and by ourselves for the mussel. Fresh oyster sperm were found by Nelson to increase markedly the rate of water propulsion by male oysters, while in females no response was observed unless spawning occurred, in which case the filtration-rate was temporarily reduced.

Parker (1914) reports on the strength and volume of water currents produced by sponges. He measured the average height to which the excurrent stream of water might reach when glass tubes of appropriate size were tied securely into an osculum. By measuring the rate at which carmine particles, etc., were carried out of a glass tube of known dimensions, Parker concluded that the sponge *Spinosella* discharged water from its oscula at a rate of about 4.5 cc. in five seconds, or about 78 liters per day. A colony of *Spinosella* having as many as twenty oscula might, he concluded, strain in a day more than 415 gallons of water.

Damas (1935) studied the activities of plankton-feeders, especially *Cardium* and other lamellibranch mollusks, with reference to their rôle in the deposition of marine muds. He calculated, on the basis of quantitative observations of the extraordinary rate of deposition of mud pellets by *Cardium*, that 1,000 such individuals produce, on an average, a layer of mud of 0.45 meter in thickness per square meter per year, or 1,250,000 cubic meters of mud per year in the 250 hectares (1 hectare = 10,000 sq. m.; 2.471 acres) colonized by *Cardium* in the roadstead of Zeebrugge.

The question of the natural filtration rate has been approached by Dodgson (1928) and by ourselves under conditions which would seem to simulate more closely those of nature. Dodgson and co-workers, working with the bay mussel, *Mytilus edulis*, at Conway, Wales, prepared turbid suspensions of different substances such as flour, clay, fine silt, and even ordinary muddy river water, in sea water. On the basis of many experiments, Dodgson claims that "end-points," i.e. the time at which the formerly cloudy solutions become quite limpid, could be determined without difficulty, the last traces of turbidity disappearing almost suddenly. At 17° C., mussels placed in turbid suspensions of fine silt,

mud, clay, or flour filtered the water to clarity at estimated minimum rates varying between 1.9 and 2.6 liters per hour per mussel. His average figure of about 2 liters per hour per mussel is expressed as being probably far less than the actual quantity, since, in order to remove quantitatively a suspended substance from a given mass of water, the animal must, because of the constant mixing of the filtered with the unfiltered water, pass some of the water through its mantle cavity many times over, even should the water issuing from the exhalant siphon be completely cleared.

Experimental

In our work with the California sea mussel, *Mytilus californianus*, which unlike the *M. edulis* that inhabits bays, estuaries and river mouths, attaches itself to rocks and pilings near open, unprotected shores, we used some refinements of Dodgson's general method. We considered that serial measurements of a finely divided substance remaining suspended in a given mass of water in which mussels were immersed should provide approximate data regarding the rate of filtration of the water by the animals.

We have not overlooked the fact that individual animals may vary considerably in their relative rates of propelling water through their gill chambers; we have also considered the possibility that the rate of filtering by the animal may depend to a considerable extent upon such factors as (*a*) particle size, (*b*) concentration and (*c*) chemical nature of the chosen suspended material (i.e. whether of nutritional, inert, or injurious character), and the possible influence of such properties upon mucus secretion, ciliary motion, and frequency of closure of the valves.

For convenience in analyzing at intervals remaining suspended material, and in order to duplicate to some extent the conditions of nature, use was made of a calcareous marine mud, whitish to light grey in color, from Bird Key Harbor, Florida. In preliminary experiments [2] use was made of material which, according to Dr. E. M. Thorp, had passed through a 0.48-mm. mesh screen. Its introduction into water in which mussels were immersed had no perceptible influence upon the animals, which remained with valves apart and continued to filter water. Mussels when kept out of water for a short interval, then placed in a very turbid suspension of the mud, opened their valves without delay, and began "pumping" water. Figures 1–4 show a series of photographs illustrative of the rather striking clarification of turbid water in a relatively short time. Into each of two graduated cylinders, each containing

[2] These were undertaken in the early summer of 1935 by the senior author in collaboration with Dr. Roderick Craig of the Division of Entomology and Parasitology, University of California.

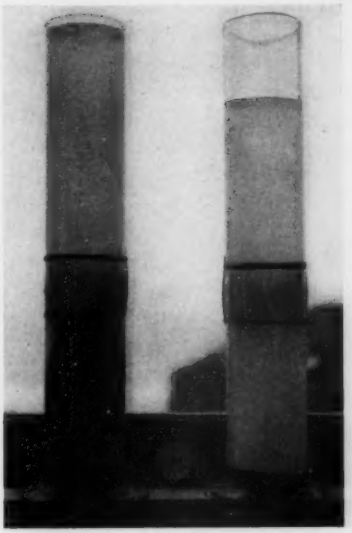

FIG. 1.

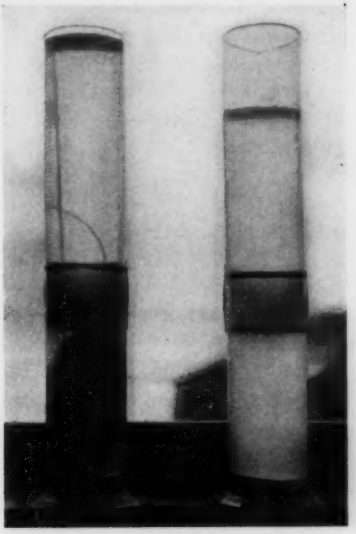

FIG. 2.

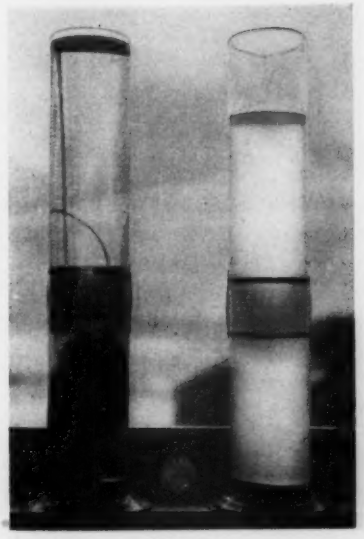

FIG. 3.

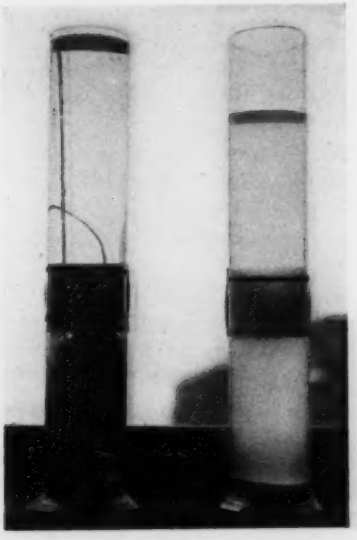

FIG. 4.

FIGS. 1–4. Removal of mud from suspension by a mussel. (Photographs by Dr. R. Craig.)

2,000 cc. of sea water, were placed 20 grams of the calcareous mud which was mixed with the water by several inversions of the temporarily stoppered containers. After the coarsest particles had settled out within a few minutes, the containers were placed in a window to afford good lighting, and a large mussel of 17 cm. length, and weighing about 485 grams, was placed in the one on the left side. The first photograph shows an identical degree of turbidity in the two suspensions at the beginning, as noted in the faint Tyndall beam from the edges of the cards strapped at the rear outer wall of each cylinder. In half an hour (Fig. 2), the turbidity in the mussel-containing jar had been greatly decreased, and the picture shows the printed letters upon the card, easily legible through the water. In 50 minutes (Fig. 3) the turbidity in the left container was all but gone, and in the last of the series (1 hour and 45 minutes) we see a perfectly clear solution. Note particularly the serial appearances of the string by means of which the animal had been lowered slowly into the container. The temperature of the collected sea water was initially about 20° to 21° C., and could have increased by not more than a degree or two.

Inspection of the sediment at the bottom of containers on the day following a filtration experiment revealed three types of precipitated mud: (1) very fine, homogeneous powder which had settled out by gravity; (2) piles of pseudofeces in the form of amorphous, stringy masses of material which had been filtered out by the mussel, incorporated in mucus, and expectorated from the mantle at the excurrent siphon; the extrusion of this material could be observed continuously from the beginning of the experiment; (3) true characteristic feces in the form of short, discrete, flattish straps composed of the mud which had been ingested by the mussel.

The intricate arrangements possessed by lamellibranchs for filtering the fine detritus and small organisms from water have been described and discussed extensively by other authors (Moore, 1905; Allen, 1914; Kellogg, 1915; Dodgson, 1928; Yonge, 1928; and others cited especially by the latter).

We know from the experiments reported in this paper and from previous ones (Fox et al., 1936) that the material filtered by the mussel from water may be in part swallowed (even if inert and nutritionally useless) and in part expectorated in mucus-laden strings or pseudofeces from the edges of the mantle, especially if the water contains a great amount of suspended matter. We were not in this particular study interested primarily in the relative quantities that were swallowed or rejected; we were interested in the rate of diminution of suspended material, and made preliminary measurements [3] in order to learn (1) how

[3] In the late summer of 1935 by the senior author jointly with Mr. Rae Schwenck, from the Department of Chemistry, Sacramento Junior College.

rapidly water may be propelled through the gill chamber of the mussel during such metabolic activities as feeding, respiration and excretion, and (2) how rapidly suspended organisms or other material may be removed from water, to be ultimately deposited in an altered state upon the bottom of the sea.

The results of these preliminary experiments were, however, not entirely satisfactory. Their difficulty of interpretation was doubtless due to the fact that we were at that time unaware of all the precautions which had to be taken in order to obtain results which could be readily analyzed.

The experiments were therefore repeated in the fall of 1936 (D. L. F. and J. P. C.), with the introduction of several refinements in technique and materials; these experiments will here be dealt with more fully. In the first of them the same calcareous mud was employed, but of a much finer grade, having passed (according to Dr. Thorp) through a 0.086 mm. mesh screen. The dry mud was shown by analysis to contain 1.66 per cent moisture, 90 per cent $CaCO_3$, and 6 per cent SiO_2 (by difference). In later experiments, we used pure $CaCO_3$ instead, and found that it served equally well. (See below.)

In our experiments 8,000 cc. of fresh sea water, to which were added initially 32 grams of mud (4 grams per liter of water), were placed in each of a series of large battery jars. The suspensions were allowed to stand for about one hour, to allow time for larger aggregates of mud particles to settle to the bottom.

The mussels were handled carefully throughout the work; they were placed on the table for a time with the valve openings vertical to the surface, in order to allow the water within the mantle cavity to drain out when the animals opened their shells; this not only prevented changes in the volume of water introduced into the containers, but probably rendered the animals sufficiently "thirsty" to insure their commencing activities almost directly they were immersed in the suspensions; the prompt opening of their valves after immersion could be recognized by bubbles of displaced air rising to the surface, and often also by watching the animals which lay close to the glass walls of the jars. A moderate stream of air was introduced through a glass tube reaching to the bottom of each jar, sufficient to insure constant homogenous mixing without disturbing the animals or stirring the heavier material which was deposited on the bottom by ordinary settling or by the animals.

Although the animals kept their valves apart during the course of the experiments, it was observed, by following the course of suspended particles, that the currents of water flowing into the incurrent and out of the excurrent siphon were not of continuous intensity, but occurred

at apparently rhythmical intervals, the intensity of the stream alternately increasing to a maximum, then gradually diminishing to a mini-

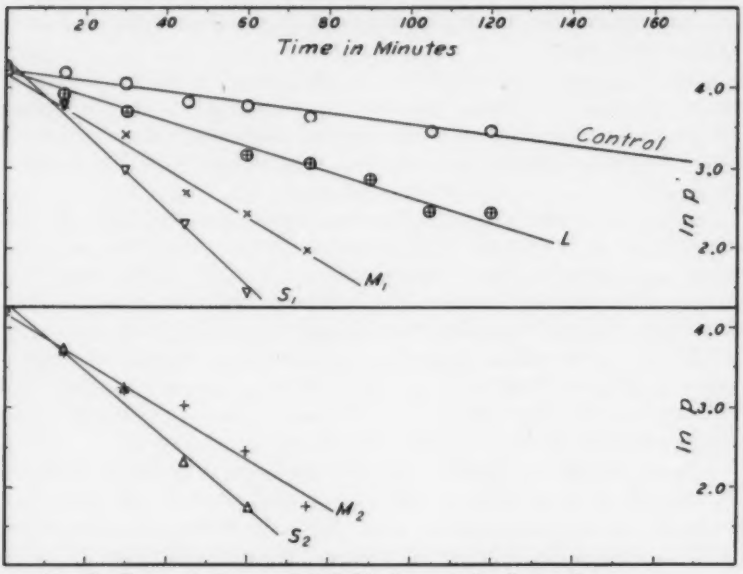

FIG. 5. Removal of suspended $CaCO_3$ by mussels; first series.

First Series:

Large mussels— 2 animals. Lengths 130 mm. and 110 mm. (average = 120 mm.).
Total weight 333 grams (average = 166.5 grams) each.

Medium mussels—(M_1) 4 animals. Lengths 97, 100, 100 and 103 mm. (average = 100 mm.).
Total weight 302.5 grams (average = 75.6 grams) each.

(M_2) 4 animals. Lengths 95, 95, 105 and 105 mm. (average = 100 mm.).
Total weight 299.4 grams (average = 74.8 grams) each.

Small mussels—(S_1) 9 animals. Lengths 70, 78, 67, 73, 76, 72, 75, 74 and 65 mm. (average = 68.9 mm.).
Total weight 308.4 grams (average = 34.4 grams) each.

(S_2) 9 animals. Lengths 78, 69, 79, 75, 82, 79, 60, 62 and 82 mm. (average = 74 mm.).
Total weight 360.0 grams (average = 40 grams) each.

Ten-cc. samples taken every 15 minutes. Temperature range 22.85° to 23.4° C.

mum. This could be observed particularly well in the large mussels. Mucus-laden strands of the filtered mud were expelled at a slow but

nearly constant rate from the edges of the mantle at the exhalant opening. These pseudofeces either fell rapidly to the bottom, or, if attached to an air bubble or two, rose to the top. They did not break down and become redispersed.

Samples were removed at stated intervals (10 or 15 minutes) from a uniform place in the center of the container and at about the mid-depth point, with calibrated 10-ml. pipettes, and introduced into stoppered vessels for analysis. These samples were acidified before washing them into containers for analysis, in order to insure that none of the colloidal $CaCO_3$ material remained adsorbed to the walls of the vessels. The microchemical method of Kirk and Moberg (1933) for the analysis of

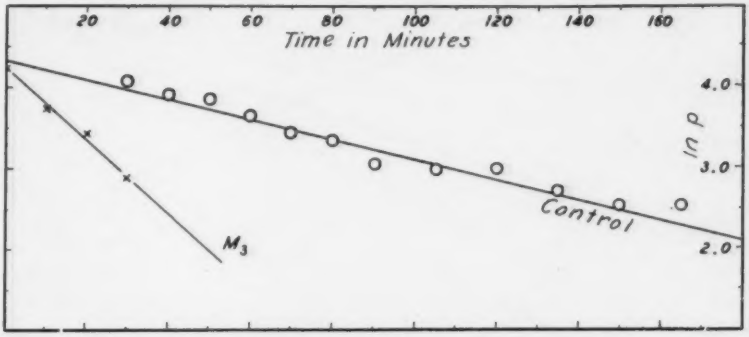

FIG. 6. Removal of suspended $CaCO_3$ by mussels; second series.

Second Series:
Six mussels used.
Medium (M). Lengths 98, 108, 100, 103, 105, 107 mm. (average = 103.5 mm.).
Total weight 611.5 grams (average = 102 grams).
Ten-cc. samples taken. Temperature range 20.8° to 21.35° C.

calcium in blood or sea water was employed. The calcium is precipitated as the oxalate, washed, and titrated in acid solution with standard potassium permanganate. By this method the amount of calcium is determined with an accuracy of ± 4 mg./liter. The concentration of dissolved calcium in the ordinary sea water used in these experiments was determined by the same method.

Controls (i.e. jars containing identical quantities of water and suspended mud, with similar moderate aeration but without mussels) were always carried out, parallel with the experiments, and samples were taken from these at the same intervals.

Two series of experiments (Numbers 1 and 2) were undertaken using the calcareous marine mud; the results are shown in Figs. 5 and 6.

The data for these figures were collected and originally tabulated in exactly the way shown in Table III.

Two series of experiments (Numbers 3 and 4) were also performed with the use of pure, finely divided $CaCO_3$; the results are recorded in Figs. 7 and 8 (from data tabulated as in Table III, which shows data from which Fig. 8 was derived). Freshly collected specimens were

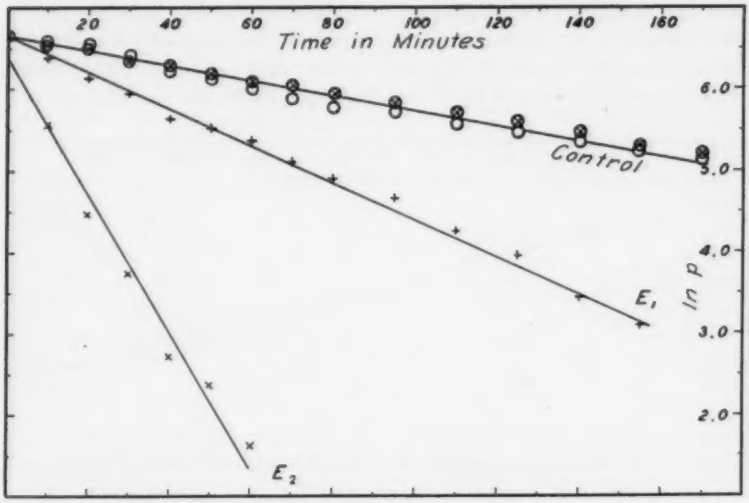

FIG. 7. Removal of suspended $CaCO_3$ by mussels; third series.

Third Series:

Very large mussels.

E_1. Two animals. Lengths 179 and 174 mm. (average = 176.5 mm.). Weights 434.7 and 395 grams respectively (average = 415 grams).

E_2. Two animals. Lengths 182 and 178 mm. (average = 180 mm.). Weights 337 and 400 grams respectively (average = 368 grams).

Ten-cc. samples taken. $CaCO_3$ used; 2 grams/liter (16 grams total) in each tank. Temperature range 18°–20° C.

used in these experiments. Placed carefully in the containers, they soon opened their valves, and showed no objection whatever when the carbonate was added, but continued to filter the water in a normal manner.

In one experiment (see Fig. 7) two pairs of very large mussels, collected at an exceptionally low tide, were employed. These individuals were selected from the catch on the basis of fair uniformity of size and their readiness to open their valves and propel water without showing

cessation of activity or other disturbed responses to slight mechanical stimuli such as stirring the water in which they were immersed, or tapping upon the container.

Four jars were set up, each containing 6 liters of sea water. Two selected mussels were placed in each of two jars while the other jars were left as controls. Air was passed at a uniform rate through each jar of water as before. After all four of the animals had parted their valves, the additional two liters of water were added to each jar (to make up the total of 8 liters), the suspended $CaCO_3$ being added with the water in the operation.

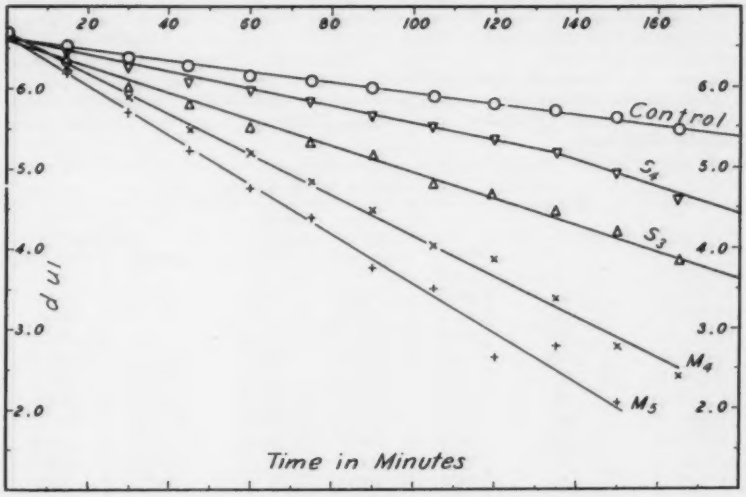

FIG. 8.

The " zero points " of the two control jars (without mussels), which agree very closely, were obtained by taking samples immediately after adding the $CaCO_3$ in the regular manner and are taken to serve as initial points for the duplicate experiments as well, since identical quantities of the salt had been added to the same volumes of water under the same conditions, and previous experiments had shown good checks in the initial quantities of suspended material under such conditions.

Table III shows data relating to duplicate experiments on (1) medium-sized mussels (M_4 and M_5) and (2) small mussels (S_3 and S_4) all run simultaneously along with a control (C). Four mussels were placed in each jar, and the experiments were conducted in a manner

identical to the previous set. Again, the initial value for suspended $CaCO_3$ in the *control* jar was taken to represent a reliable figure for that of the experimental jars.

In these experiments wherein $CaCO_3$ was used, it is noted that although the initial quantity of $CaCO_3$ suspended in the water exceeds by an average of some ten-fold the quantity suspended when the calcareous marine mud was employed in the earlier experiments, nevertheless the

TABLE III

Removal of suspended $CaCO_3$ by mussels—fourth series. Medium mussels: M_4—4 animals. Lengths 105, 100, 102 and 109 mm. (Average = 104 mm. each). Total weight 342.5 grams (Average = 85.6 grams each). M_5—4 animals. Lengths 106, 104, 102 and 108 mm. (Average = 105 mm. each). Total weight 337.5 grams (Average = 84.4 grams each). Small mussels: S_3—4 animals. Lengths 78, 79, 77 and 74 mm. (Average = 77 mm. each). Total weight 139 grams (Average = 34.75 grams). S_4—4 animals. Lengths 75, 81, 74 and 77 mm. (Average = 77 mm. each). Total weight 156 grams (Average = 39 grams each). Ten-cc. samples taken. Temperature range 19°–20.3° C. $CaCO_3$ used. Two grams per liter (Sixteen grams total in each tank).

Time	Minutes	Control C	Medium M_4	Medium M_5	Small S_3	Small S_4
		Ca	Ca	Ca	Ca	Ca
		grams/liter	*grams/liter*	*grams/liter*	*grams/liter*	*grams/liter*
1:15 P.M....	0	1.2356	—	—	—	—
2:10 P.M....	15	1.1185	.9542	.9314	1.0142	1.1000
2:25 P.M....	30	1.0171	.7828	.7271	.8385	.9542
2:40 P.M....	45	.9585	.6714	.6142	.7571	.8642
2:55 P.M....	60	.8928	.6057	.5457	.6771	.8214
3:10 P.M....	75	.8685	.5543	.5114	.6371	.7642
3:25 P.M....	90	.8242	.5185	.4728	.6000	.7200
3:40 P.M....	105	.7828	.4871	.4628	.5500	.6814
3:55 P.M....	120	.7528	.4771	.4443	.5343	.6428
4:10 P.M....	135	.7300	.4585	.4457	.5143	.6085
4:25 P.M....	150	.7028	.4457	.4385	.4957	.5671
4:40 P.M....	165	.6685	.4414	.4243	.4771	.5314
4:55 P.M....	180	.6500	.4343	.4257	.4685	.5143

rate of removal of suspended material by mussels of from 69 to 105 mm. in length is similar in both series of experiments. (See Table IV.)

The total calcium content represents the sum of the amount of calcium which is present in solution in the sea water and the added amount which is present in suspended material. The decrease of the calcium content with time is due to:

1. Gravitational settling of suspended material.
2. Removal (filtration) of suspended material by the mussels.

In the control experiments, which were undertaken without mussels in the vessel, the decrease was due to gravitational settling only, and a

general law can be found for the rate of this settling. During the experiments with mussels in the vessel, the decrease was due partly to settling and partly to removal of the material from suspension by the mussels; knowing the former, the rate of removal of the calcium by the mussels can be determined by difference. In all cases the mussels decreased the calcium content to a minimum value of about 430 mg. Ca per liter, whereas a determination of the contents in water filtered through paper gave about 440 mg. Ca per liter. Additional unpublished experiments have shown that a small amount of the calcium in the water, present probably in a very finely suspended state, could be filtered by the mussels but was not filtered out by paper alone, previous to analysis. It

TABLE IV

Liters of water passed per mussel per hour.

Series No. and value of a	Experiment No.	Size of mussel		Number of mussels	Value of b	m in liters per hour
		Mean weight	Mean length			
		grams	mm.			
(1) 0.39	1	166.5	120	2	0.93	2.2
	2	75.6	100	4	1.81	2.8
	3	74.8	100	4	1.82	2.9
	4	34.3	69	9	2.80	2.1
	5	40.0	74	9	2.57	1.9
(2) 0.70	6	101.9	103.5	6	2.59	2.5
(3) 0.54	7	415	176.5	2	5.05	18.1
	8	368	180	2	1.36	3.3
(4) 0.41	9	85.6	104	4	1.51	2.2
	10	84.4	105	4	1.85	2.9
	11	34.8	77	4	0.98	1.1
	12a	39.0	77	4	0.65	0.5
	12b	39.0	77	4	1.02	1.2

appears therefore justifiable to assume that the content in solution was about 430 mg. Ca per liter and that *the suspended amount was equal to the observed value minus 430 mg. Ca per liter.* The value 430 may be in error by about 1 mg./liter whereby a slight uncertainty is introduced as to the amount of suspended calcium, and this uncertainty exerts a corresponding slight influence upon the further treatment of the data, since this has to be based upon the *suspended* amount of calcium and not upon the total amount.

By plotting the results from the control experiment one obtains a curve of a form which suggests that in the absence of mussels the amount of suspended calcium can be expressed as an exponential func-

tion of time. We assume, therefore, that the amount which is precipitated in unit time is proportional to the total amount which is suspended. If we have in the vessel M liters of water containing p mg. of suspended calcium per liter, the amount precipitated in unit time will be aMp, where a is a factor of proportionality. The amount precipitated in the time dt will be:

$$(a\ M\ p\ dt)$$

Concerning the action of the mussel, we will assume that *in unit time each mussel pumps* m *liters of water through its system and removes all suspended calcium from this quantity of water.* The amount removed by one mussel in unit time will then be $m\ p$, and if we have n mussels the amount of suspended calcium removed in the time dt will be:

$$(n\ m\ p\ dt)$$

The total amount removed owing to both ordinary precipitation and to the action of the mussels, representing the total decrease of the suspended amount of calcium, is therefore:

$$d(M\ p) = -\ (nm + aM)\ p\ dt \tag{1}$$

where the minus sign indicates a decrease. Therefore;

$$\frac{dp}{p} = -\left(\frac{nm}{M} + a\right) dt = -\ bdt$$

$$\ln p = -\left(\frac{nm}{M} + a\right) t = -\ bt \tag{2}$$

$$p = p_0 e^{-\left(\frac{nm}{M} + a\right)t} = p_0 e^{-bt} \tag{3}$$

The logarithmic decrement a can be determined from the control experiment and the logarithmic decrement b from the experiments with mussels. The amount of water which passes through one mussel in unit time is then:

$$m = M\,\frac{b - a}{n} \tag{4}$$

If our assumptions are correct, each experiment must reveal a linear relationship between the natural logarithm of p (the amount of suspended calcium in mg./liter) and the time. Furthermore, we must obtain nearly the same values of p_0, the amount of suspended calcium at the beginning of the experiment, since in each case the initial conditions were as similar as possible.

In order to examine this question, the values of p have been computed by subtracting 430 mg./liter from the observed values of the total calcium content. All values of p smaller than 5 mg./liter have been omitted (except in one instance when a value of 4.3 mg./liter has been retained) since, owing to the slight uncertainty involved in the micro method for determining the amount in solution (430 mg./liter), small values of p have no significance. In the case of the control experiments values only from the first three hours have been considered, although p remained greater than 5 mg./liter.

In Figs. 5, 6, 7, and 8, the values of $ln\ p$, as derived from the four series of experiments, have been plotted against time. The straight lines represent lines of regression which have been computed by the method of least squares. It is seen that all points fall so near to the lines that the deviations may be considered accidental. Accidental deviations must arise, since the samples taken from the middle of the jar cannot be expected to show exactly the average contents of suspended calcium, and since errors of determination are present. In the case of the small mussels (S_4), in series No. 4, a break in the line appears to occur after 130 minutes, indicating that the rate of propulsion of water through the mussels suddenly changed.

From the control experiments, we obtain various values of our constant a, using one hour as the unit of time (see Table IV). The variation of the values of a may be due to differences in the velocity of the stream of air which was passed through the water in order to insure homogeneous mixing, or, to a lesser extent, to differences in the effect of the salt water in coagulating the suspended material. In all experiments, however, the velocity of the air stream was adjusted so that the rate of bubbling through control jars and experimental jars was as closely identical as estimation would permit.

From the experiments with mussels we find the values of b which are shown in Table IV and the values of m (in liters per hour) which have been computed by means of formula (4), introducing M (8 liters), the number of mussels (n), and the value of a which was found by simultaneous control experiment.

The fact that in all cases we find a linear relationship between $ln\ p$ and time appears to furnish strong evidence for the correctness of our assumptions, but from our equations it is evident that we should obtain a linear relationship on other assumptions than these which have been introduced. We could assume that the mussels remove only a constant fraction of the suspended calcium when m liters pass through the mussel. The amount removed would then be $cnm\ p$, where c must be sup-

posed to be a constant factor smaller than 1.0. It appears, however, improbable that a mussel should remove, say, 50 mg./liter when the content was 100 mg./liter and in the same interval of time, 2 mg./liter when the content was 4 mg./liter. A change in the ratio of the removed calcium from say 80 per cent of a high content to 100 per cent of a small content is, however, possible and would not be detected. If such a change takes place, our values of m are conservative, being somewhat low. Another conceivable possibility which may be mentioned is that the amount of water passing through the mussel chamber might decrease with time and the fraction of calcium removed might increase (or vice versa). However, the product mp must, since $ln\ p$ is a linear fraction of time, remain nearly constant, and this means that the amount of water passing through the mussel chamber and the fraction of calcium removed would have to change in opposite directions at the same rate. It is difficult to conceive a mechanism which would work in such a manner. The simplest explanation of our results appears to be that our original assumptions are correct, viz., that a constant amount of water passes through the mussel chamber in unit time and that virtually all suspended (and colloidal) calcium in this amount of water is removed.

We have furthermore supposed that the water passing through the mussels has the average calcium content of the water in the vessel. In order to satisfy this condition, the water must be stirred, since the mussels lying on the bottom of the vessel remove practically all suspended calcium from the water passing through them. The stirring must be adapted to the number of mussels; the greater this number the more rapid must the stirring be. The effect of insufficient stirring upon the observations would be that $ln\ p$ would no longer be a linear function of time, but would decrease more and more slowly as the experiment advanced. The reason for this is that the amount of suspended calcium removed by the mussels would no longer be proportional to p, but since the bottom layer, where the mussels lie, would be depleted of calcium more rapidly, it would be proportional to some function of p which decreases with time. We can introduce

$$nm\ p\ e^{-ct}\ dt$$

instead of

$$nm\ p\ dt$$

and obtain

$$\ln p = \ln p_0 - at - \frac{nm}{Mc}(1 - e^{-ct})$$

At great values of t, we obtain

$$\ln p = \ln p_0 - \frac{nm}{Mc} - at,$$

meaning that the decrease in the calcium content approaches the value which is due to gravitational settling only. The coefficient c which has been introduced depends upon the rate with which water passes through the mussels and upon the rate of stirring, and a determination of this coefficient is hardly possible. A computation therefore cannot be based upon the results of such experiments. During the experiments which are dealt with here, the water was stirred by passing through a constant stream of air, and the agreement between observed and computed values shows that the stirring was sufficient.

Two preliminary experiments which Fox and Schwenck undertook in the summer of 1935 with two very large mussels weighing 485 and

TABLE V

Rates of water propulsion by mussels.

Weight of mussels		Length of mussels		Number experiments	Total no. mussels used	Rate of water propulsion	
Range	Average	Range	Average			Range	Average
grams	*grams*	*mm.*	*mm.*			*liters/hr.*	*liters/hr.*
337–515	431	174–182	178	4	6	1.8–18.1	6.4
75–166	93	95–130	102	6	24	2.2– 2.9	2.6
34– 40	37	60– 82	74	4	26	0.5– 2.1	1.4

515 grams show a wider scattering of the observed values, but they indicate a linear relationship between $ln\ p$ and t, giving m equal to 1.8 and 2.4 liters per hour respectively.

Other preliminary experiments by Fox and Schwenck, in the summer of 1935, gave less consistent values. In these experiments a large number of small mussels were used and $ln\ p$ decreased rapidly at the beginning, but slowly at the end of the experiments, probably because the stirring was insufficient.

The results of all experiments, including the preliminary ones by Fox and Schwenck, are summarized in Table V. The very large mussels show an enormous range in their rate of water propulsion, perhaps owing to individual differences or perhaps because they work intermittently. The value obtained from one of the experiments, 18.1 liters per hour, is quite enormous, but it is undoubtedly correct since it is based on good observations during one full hour, and it shows that in certain cir-

cumstances large mussels can pump great quantities of water through their systems. The average value for the large mussels, 6.4 liters per hour, cannot be given any weight owing to the wide range of the single values and the small number of experiments. The medium-sized mussels appear to be more consistent in their behavior, the range of the single values is small and the average value, 2.6 liters per hour, can therefore be considered nearly correct. The small mussels show again a wider range of their rate of water propulsion and while the average value is uncertain, it undoubtedly lies below that of the larger animals.

It would be expected that both the weight and the volume (capacity of the gill chamber) of the mussel should be proportional to some fairly constant power of one of the linear dimensions, say the length of the shell, and that weight should show a linear relationship to capacity. The capacity (in ml.) was determined by allowing mussels whose gill chambers had been emptied of sea water to refill their cavities on immersion in a known volume of sea water, then causing the animals to close their valves, removing them from the vessel, and measuring the residual water in the container. Plotting the average lengths of a series of different sized animals (in mm.) against their capacities (in ml.) showed a steeply rising curve of an exponential character; length plotted similarly against weight of animals (in grams) emptied of water showed a curve of similar character (see also Galtsoff, 1931, who obtained a similar relationship in the Hawaiian pearl oyster); finally the relationship between weight and capacity was shown to be a linear one.

An extensive series of experiments performed upon mussels of different known capacities, weights, and linear dimensions would perhaps disclose an interesting relationship between any of these attributes (say the volume), and the rate of propulsion of water by each size of "pumping system."

Summary

A method is described for determining the approximate average rate at which the California mussel, *Mytilus californianus,* propels water through its gill chambers.

The method consists of analyses at frequent intervals of the amount of calcium remaining in suspension (as $CaCO_3$) in a given volume of continually stirred water containing the mussels, which remove the suspended material as they pass the water through their chambers.

Mathematical treatment and interpretation of the data obtained, support the conclusions that (1) virtually all of the suspended matter is removed as the water passes over the mucous surfaces of the gills and mantle of the mussel; (2) the mussel propels the water rhythmically

through its filtering system at a rate which on an average is constant, varying according to the size and perhaps also according to other physiological attributes of the animals. In medium-sized animals (of 95 to 130 mm. length) the propulsion rate may vary between extreme values of 2.2 and 2.9 liters per hour, and has an average value of approximately 2.6 liters per hour.

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INDEX

ABRAMOWITZ, ALEXANDER A. The chromatophorotropic hormone of Crustacea, 344.

Acanthephyridæ, eyes of, 57.

Accelerator substances, evidence for production by ultraviolet radiation of Limulus muscle, 75.

ALLEE, W. C., AND GERTRUDE EVANS. Some effects of numbers present on the rate of cleavage and early development in Arbacia, 217.

Amœba proteus, mitosis in, 125.

Amphiscolops langerhansi, reproductive system and copulation, 319.

Arbacia, cleavage rate, as affected by numbers present, 217.

Arbacia punctulata, prolongation of life in egg cells and calcium reduction, 366.

AREY, LESLIE B. Observations on two types of respiration in Onchidium, 41.

Aurelia, budding and locomotion in scyphistomas of, 99.

BACTERIA, and phosphorus cycle in the sea, 190.

BEADLE, G. W. *See* Clancy and Beadle, 47.

VON BRAND, THEODOR. Observations upon the nitrogen of the particulate matter in the sea, 1.

—— ——, ——, NORRIS W. RAKESTRAW AND CHARLES E. RENN. The experimental decomposition and regeneration of nitrogenous organic matter in sea water, 165.

Budding and locomotion in scyphistomas of Aurelia, 99.

CALANUS finmarchicus, feeding rate of, in relation to environmental conditions, 233.

Calcium reduction and prolongation of life in egg cells of Arbacia punctulata, 366.

CAMERON, JOHN ANDREW. The mitotic rate in tadpole skin after repeated injury, 37.

Caterpillars, gustation and olfaction in, 7.

Centrifuging, behavior of maturation spindles in polar fragments of Ilyanassa eggs after, 88.

CHACE, F. A., JR. *See* Welsh and Chace, 57.

CHASE, HYMAN Y. The effect of ultraviolet light upon early development in eggs of Urechis caupo, 377.

Chromatophorotropic hormone of Crustacea, 344.

CHURNEY, LEON, AND HERBERT M. KLEIN. The electrical charge on nuclear constituents (salivary gland cells of Sciara coprophila), 384.

Clam, adult, seasonal gonadal changes, 406.

——, development of primary gonad in, 389.

CLANCY, C. W., AND G. W. BEADLE. Ovary transplants in Drosophila melanogaster: studies of the characters singed, fused, and female-sterile, 47.

Cleavage, rate in Arbacia, effects of numbers present, 217.

Color-changes, and chromatophorotropic hormone of Crustacea, 344.

—— ——, and diurnal rhythm in Ligia baudiniana, 24.

COONFIELD, B. R. Symmetry and regulation in Mnemiopsis leidyi, Agassiz, 299.

CUNNINGHAM, JOHN P. *See* FOX, Sverdrup and Cunningham, 417.

Cyst formation in glomerular tufts of certain fish kidneys, 247.

DALCQ, A., AND G. VANDEBROECK. On the significance of the polar spot in ripe unfertilized and in fertilized ascidian eggs, 311.

DAWSON, J. A., WALTER R. KESSLER AND JOSEPH K. SILBERSTEIN. Mitosis in Amœba proteus, 125.

Decomposition, experimental, nitrogenous organic matter in sea water, 165.

Depressor substances, production by ultraviolet radiation of Limulus muscle, 75.
DETHIER, VINCENT G. Gustation and olfaction in lepidopterous larvæ, 7.
Digestion, enzymes of, in Daphnia and Polyphemus, Diaptomus and Calanus, 290.
Drosophila melanogaster, ovary transplants in, 47.

ELECTRICAL charge on nuclear constituents of salivary gland cells, 384.
Embryonic determination, production of intermediate-winged aphids and, 259.
Endomixis, induction of, in Paramecium aurelia, 196.
Enzymes, digestive, in Daphnia and Polyphemus, Diaptomus and Calanus, 290.
EVANS, GERTRUDE. *See* Allee and Evans, 217.
Eyes, of Acanthephyridæ, 57.

FAURÉ-FREMIET, E. Licnophora lyngbycola, a new species of infusorian from Woods Hole, 212.
Feeding rate of Calanus finmarchicus in relation to environmental conditions, 233.
Food, effects on gastric epithelial cells of grasshopper, Melanoplus differentialis Thomas, 203.
——, rate of locomotion and reproduction in marine Amœba, 334.
FOX, DENIS L., H. U. SVERDRUP AND JOHN P. CUNNINGHAM. The rate of water propulsion by the California mussel, 417.
FULLER, JOHN L. Feeding rate of Calanus finmarchicus in relation to environmental conditions, 233.

GASTRIC epithelial cells, effects of diet, in grasshopper, 203.
GILCHRIST, FRANCIS G. Budding and locomotion in the scyphistomas of Aurelia, 99.
Gonad, changes of, seasonal, in adult clam, 406.
——, primary, development in Venus mercenaria Linnæus, 389.
Gorgodera amplicava, development of, in final host, 80.
GRAFFLIN, ALLAN L. Cyst formation in the glomerular tufts of certain fish kidneys, 247.
Grasshopper, effects of diet on gastric epithelial cells of, 203.
Gustation and olfaction in lepidopterous larvæ, 7.
GUTTMAN, S. A. Evidence for the production of accelerator and depressor substances by ultraviolet radiation of Limulus muscle, 75.

HASLER, ARTHUR D. The physiology of digestion in plankton Crustacea. II. Further studies on the digestive enzymes of (A) Daphnia and Polyphemus. (B) Diaptomus and Calanus, 290.
HODGE, CHARLES, 4TH. Some effects of diet on the gastric epithelial cells of the grasshopper, Melanoplus differentialis Thomas, 203.
HOPKINS, DWIGHT LUCIAN. The relation between food, the rate of locomotion and reproduction in the marine Amœba, Flabellula mira, 334.
Hormone, chromatophorotropic, of Crustacea, 344.
Hydroid, new genus and method of asexual reproduction, 327.
HYMAN, LIBBIE H. Reproductive system and copulation in Amphiscolops langerhansi (Turbellaria Acœla), 319.

ILYANASSA eggs, behavior of maturation spindles in polar fragments of, after centrifuging, 88.
Injury, mitotic rate in tadpole skin after, 37.
Intermediate-winged aphids and embryonic determination, 259.

KESSLER, WALTER R. *See* Dawson, Kessler and Silberstein, 125.
Kidneys, fish, cyst formation in glomerular tufts of, 247.
KLEIN, HERBERT M. *See* Churney and Klein, 384.
KLEINHOLZ, L. H. Studies in the pigmentary system of Crustacea. I. Color changes and diurnal rhythm in Ligia baudiniana, 24.
——, —. —. Studies in the pigmentary system of Crustacea. II. Diurnal

movements of the retinal pigments of Bermudan decapods, 176.

LICNOPHORA lyngbycola, new species of infusorian from Woods Hole, 212.
Ligia baudiniana, color changes and diurnal rhythm, 24.
Locomotion and budding in scyphistomas of Aurelia, 99.
——, rate of, reproduction, and food in marine Amœba, 334.
LOOSANOFF, VICTOR L. Development of the primary gonad in Venus mercenaria Linnæus, 389.
——, ——, —. Seasonal gonadal changes of adult clams, Venus mercenaria (L.), 406.
Loping of land-snails, 287.

MATURATION spindles, behavior of, in polar fragments of eggs of Ilyanassa obtained by centrifuging, 88.
MILES, SAMUEL STOCKTON. A new genus of hydroid and its method of asexual reproduction, 327.
Mitosis in Amœba proteus, 125.
——, rate of, in tadpole skin after repeated injury, 37.
Mnemiopsis, symmetry and regulation, 299.
MORGAN, T. H. The behavior of the maturation spindles in polar fragments of eggs of Ilyanassa obtained by centrifuging, 88.
Muscle, Limulus, production of accelerator and depressor substances by ultraviolet radiation, 75.

NITROGEN of particulate matter in the sea, 1.
Nitrogenous organic matter in sea water, experimental decomposition and regeneration, 165.
Nuclear constituents, electrical charge on, in salivary gland cells, 384.

ODLAUG, THERON O. Notes on the development of Gorgodera amplicava in the final host, 80.
Olfaction in lepidopterous larvæ, 7.
Onchidium, two types of respiration in, 41.
Ovary transplants in Drosophila melanogaster, 47.

PARAMECIUM aurelia, induction of endomixis, 196.
PARKER, G. H. The loping of land-snails, 287.
Phosphorus cycle and bacteria in the sea, 190.
Pigments, retinal, diurnal movements of, Bermudan decapods, 176.
Polar spot, significance of, in ripe unfertilized and in fertilized ascidian eggs, 311.

RAKESTRAW, NORRIS W. *See* von Brand, Rakestraw and Renn, 165.
Regulation and symmetry in Mnemiopsis leidyi, 299.
RENN, CHARLES E. Bacteria and the phosphorus cycle in the sea, 190.
——, —— —. *See* von Brand, Rakestraw and Renn, 165.
Reproduction, asexual, in new genus of hydroid, 327.
——, cycles of, and superfetation in poeciliid fishes, 145.
——, relation between food, locomotion rate and, in Amœba, 334.
——, system of, and copulation in Amphiscolops langerhansi, 319.
Respiration, two types, in Onchidium, 41.
Rhythm, diurnal, and color changes in Ligia baudiniana, 24.

SALIVARY gland cells, electrical charge on nuclear constituents, 384.
SCHECHTER, VICTOR. Calcium reduction and the prolongation of life in the egg cells of Arbacia punctulata, 366.
Sea water, nitrogenous organic matter in, experimental decomposition and regeneration of, 165.
SHULL, A. FRANKLIN. The production of intermediate-winged aphids with special reference to the problem of embryonic determination, 259.
SILBERSTEIN, JOSEPH K. *See* Dawson, Kessler and Silberstein, 125.
Snails, land, loping of, 287.
SONNEBORN, T. M. Induction of endomixis in Paramecium aurelia, 196.
Superfetation and reproductive cycle in poeciliid fishes, 145.
SVERDRUP, H. U. *See* Fox, Sverdrup and Cunningham, 417.

Symmetry and regulation in Mnemiopsis leidyi, 299.

TURNER, C. L. Reproductive cycles and superfetation in poeciliid fishes, 145.

ULTRAVIOLET light, effect on early development in eggs of Urechis caupo, 377.

—— radiation, production of accelerator and depressor substances, in Limulus muscle, 75.

Urechis caupo, eggs of, effect of ultraviolet light, 377.

VANDEBROECK, G. *See* Dalcq and Vandebroeck, 311.

WATER propulsion, rate of, by California mussel, 417.

WELSH, J. H., AND F. A. CHACE, JR. Eyes of deep sea crustaceans. I. Acanthephyridæ, 57.

Volume LXXII Number 3

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69

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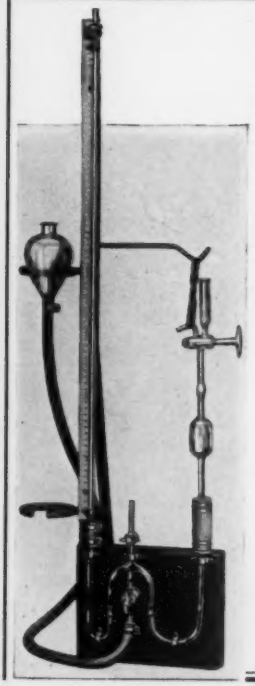

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CONTENTS

Page

SHULL, A. FRANKLIN
The Production of Intermediate-winged Aphids with Special Reference to the Problem of Embryonic Determination 259

PARKER, G. H.
The Loping of Land-snails 287

HASLER, ARTHUR D.
The Physiology of Digestion in Plankton Crustacea. II. Further studies on the digestive enzymes of (A) Daphnia and Polyphemus. (B) Diaptomus and Calanus 290

COONFIELD, B. R.
Symmetry and Regulation in Mnemiopsis leidyi, Agassiz 299

DALCQ, A., AND G. VANDEBROECK
On the Significance of the Polar Spot in Ripe Unfertilized and in Fertilized Ascidian Eggs 311

HYMAN, LIBBIE H.
Reproductive System and Copulation in Amphiscolops langerhansi (Turbellaria Acoela) 319

MILES, SAMUEL STOCKTON
A New Genus of Hydroid and its Method of Asexual Reproduction 327

HOPKINS, DWIGHT LUCIAN
The Relation between Food, the Rate of Locomotion and Reproduction in the Marine Amoeba, Flabellula mira 334

ABRAMOWITZ, ALEXANDER A.
The Chromatophorotropic Hormone of the Crustacea 344

SCHECHTER, VICTOR
Calcium Reduction and the Prolongation of Life in the Egg Cells of Arbacia punctulata 366

CHASE, HYMAN Y.
The Effect of Ultra-violet Light upon Early Development in Eggs of Urechis caupo 377

CHURNEY, LEON AND HERBERT M. KLEIN
The Electrical Charge on Nuclear Constituents (Salivary Gland Cells of Sciara coprophila) 384

LOOSANOFF, VICTOR L.
Development of the Primary Gonad and Sexual Phases in Venus mercenaria Linnaeus 389

LOOSANOFF, VICTOR L.
Seasonal Gonadal Changes of Adult Clams, Venus mercenaria (L.) 406

FOX, DENIS L., H. U. SVERDRUP AND JOHN P. CUNNINGHAM
The Rate of Water Propulsion by the California Mussel 417